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INFLUENCE OF GALACTOFLAVIN AND INANITION ON THE ADRENAL ASCORBIC ACID RESPONSE TO STRESS IN RATS[1]

GRANT G. SLATER[2]

Department of Physiological Chemistry, School of Medicine, University of California Medical Center, Los Angeles, California

ABSTRACT

Rats on a riboflavin-deficient diet containing the riboflavin antagonist, galactoflavin, lost the ability to respond to the stress of unilateral adrenalectomy, as measured by the decrease in ascorbic acid (AAA) in the contralateral adrenal. This impairment was noted in 10 days at an initial body weight of 80 gm., 22 days at 100 gm., and 35 days at 200 gm. Additional stresses such as loss of the tail and removal of 0.5 ml of blood were likewise incapable of causing a decrease in AAA in the deficient animals. However, extensive laparotomy did result in a decrease in AAA, suggesting that the pituitary-adrenocortical system in these animals was still responsive to severe stress.

The impairment in adrenal response was noted in rats maintained on both high carbohydrate and high protein diets containing galactoflavin and deficient in riboflavin. However, pair-weighed controls receiving a complete, galactoflavin-free diet also exhibited an impaired response. Riboflavin injection before unilateral adrenalectomy did not restore the response of the deficient animals. Feeding a complete diet for two days to riboflavin-deficient rats restored the response as did *ad libitum* feeding of the riboflavin-deficient diet to pair-weighed control rats for three days. Weanling rats placed on high carbohydrate riboflavin-deficient diets for 82 days gave a significant decrease in AAA to unilateral adrenalectomy. Although unpublished results from this laboratory and published results by Forker and Morgan indicate that riboflavin is essential for the release of AAA under certain conditions, the data in this paper suggest that dietary factors other than riboflavin may be more important in the AAA response to stress.

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[2] Present address: Department of Psychiatry.

FORKER and Morgan (1) demonstrated that rats maintained on a riboflavin-deficient diet lost their ability to respond to the stress of anoxia with a depletion of adrenal ascorbic acid (AAA). Since the AAA response to corticotropin (ACTH) was retained, these authors concluded that the biochemical lesion responsible for this phenomenon was located in the brain or pituitary rather than in the adrenal cortex. The inability to respond to stress with a depletion of AAA was established in about 11 weeks in animals on a riboflavin-deficient diet and was not observed in pair-weighed rats (1).

Various riboflavin derivatives, including galactoflavin, inhibit the utilization of this vitamin (2, 3, 4, 5). It seemed of interest to determine whether the pituitary-adrenal system exhibited a reduced responsiveness to stress in animals made riboflavin-deficient by feeding galactoflavin. The results reported below demonstrate that, under these circumstances, adrenal refractoriness, as measured by AAA depletion, could be established in 10 days as compared to 11 weeks in animals on a riboflavin-deficient diet alone. On the other hand, impairment of the AAA response could also be produced in pair-weighed rats consuming restricted amounts of a complete diet.

METHODS

Male Sprague-Dawley rats from our own colony, varying in weight from 50 to 200 gm., were maintained prior to use on Purina laboratory chow which contained 75 parts per million of riboflavin. Thereafter, they were placed on one of the riboflavin-free or galactoflavin diets described below. The riboflavin-free diets employed were 1) a high carbohydrate diet used for riboflavin assay[3] consisting of 68% carbohydrate, 18% protein, 10% vegetable oil, and 4% U.S.P. salt mixture, or 2) a high protein diet consisting of 72% protein, 20% vegetable oil and 8% salt mixture with all vitamins at 4 times the level found in the carbohydrate diet. Galactoflavin was incorporated into these diets at a level of 1 mg. per gm. of diet. Riboflavin supplementation of the deficient diets was made at a level of either 0.1 or 0.4 mg. per gm. of diet. Water was given to the animals *ad libitum*.

In most instances, the stress applied consisted of removal of the left adrenal with a minimum of trauma under sodium pentobarbital (Nembutal) anesthesia (4–5 mg. per 100 gm. of body weight). One hour later the right adrenal was removed using ethyl ether anesthesia if the animal had recovered from Nembutal. Ascorbic acid was determined in each adrenal by the method of Roe and Kuether (6).

RESULTS

Influence of age on the rate of disappearance of the AAA response to stress in glactoflavin-fed rats. Table 1 shows that the AAA response to unilateral adrenalectomy was rapidly lost in young rats (initial weight, 80 gm.) after they were placed on the high carbohydrate, galactoflavin-containing diet. After 5 days on this diet, AAA depletion was still essentially normal in 5 animals, but was blocked in 2 others. After 10 days, the AAA no longer re-

[3] Nutritional Biochemical Co., Cleveland Ohio.

TABLE 1. CHANGE IN THE ADRENAL ASCORBIC ACID STRESS RESPONSE WITH TIME IN 80 GM. RATS[1] MAINTAINED ON A HIGH CARBOHYDRATE DIET[2] CONTAINING GALACTOFLAVIN

Days on diet	No. of rats	Final weight, gm. Ave. ±S.E.	Adrenal		
			Weight, mg. Ave. ±S.E.	% change in ascorbic acid	
				Individual values	Ave. ±S.E.
0	9	84 ±2	20.3 ±0.4	−72, −70, −69, −67, −59, −58, −58, −57, −53	−63 ±2
5	7	73 ±1	23.3 ±1.0	−60, −56, −49, −47, −34, −14, +2	−37 ±9
10	6	65 ±2	25.9 ±1.1	−11, −10, −5, +1, +2, +12	− 2 ±4
13	7	63 ±1	24.7 ±0.8	−18, −16, −12, −4, 0, 0, +17	− 5 ±5
14	5	61 ±3	23.7 ±1.6	−29, −21, −18, +3, +9	−11 ±7
15	10	56 ±3	24.2 ±0.8	−37, −37, −20, −13, −11, −10, −4, +1, +7, +15	−11 ±5
16	1	53	21.7	−14	−14
17	4	59 ±2	27.7 ±1.8	−13, −3, +2, +16	+ 1 ±6
21	3	66 ±3	26.9 ±1.9	−7, −4, −4	− 5 ±1
23	2	59 ±4	25.1 ±0.4	−19, −5	−12 ±7
25	1	50	25.8	−8	− 8
33	7	55 ±3	21.9 ±0.9		

[1] Initial body weight, 75–85 gm.
[2] Deficient in riboflavin.

sponded to this stress. Although occasional animals exhibited a response with longer periods of feeding, an impaired response was evident even after 25 days on the analog-containing riboflavin-deficient diet. Body weight declined rapidly at first, but then plateaued. Adrenal weights were generally increased. The physical appearance of these animals was not markedly altered.

In older animals more time was required to block completely the AAA response to surgical stress (Table 2): in 100 gm. rats, 22 days on the diet and in 200 gm. animals, 35 days. Adrenal weights were slightly depressed at these times. Body weight loss was considerable.

Influence of severity of stress on the AAA response to stress in galactoflavin-fed rats. Attempts were made to evoke the AAA response by increasing the severity of stress in young rats (initial weight, 80 gm.) fed the high carbohydrate galactoflavin-containing diet for 14 to 18 days.

Increasing the time interval between removal of left and right adrenals to 2 hours, or removing the tail and 0.2 to 0.6 ml. of blood, had no influence on AAA release in these animals. On the other hand, excessively severe surgical stress was capable of evoking the adrenal ascorbic acid response. In the latter experiments, animals were exposed from the sternum to the

TABLE 2. CHANGE IN THE ADRENAL ASCORBIC ACID STRESS RESPONSE WITH TIME IN 100 AND 200 GM. RATS[1] MAINTAINED ON A HIGH CARBOHYDRATE DIET[2] CONTAINING GALACTOFLAVIN

Days on diet	No. of rats	Final weight, gm. Ave. ±S.E.	Adrenals		
			Weight, mg. Ave. ±S.E.	% change in ascorbic acid	
				Individual values	Ave. ±S.E.
Group I					
0	6	103 ±2	27.1 ±1.3[3]		
14	7	79 ±2	26.0 ±0.8	−37, −36, −21, −19, −7, 0, +1	−17 ±6
18	8	71 ±2	26.7 ±1.2	−30, −27, −24, −22, −5, −3, −1, −1	−14 ±5
22	9	64 ±2	25.6 ±1.2[3]	−31, −8, −4, −3, −2, +2, +3, +5, +15	− 3 ±4
Group II					
0	27	199 ±1	35.8 ±0.5[4]		
21	8	135 ±3	35.5 ±1.4	−51, −47, −44, −41, −36, −32, −17, −4	−34 ±6
28	8	127 ±3	32.9 ±1.0	−36, −24, −20, −20, −19, −16, −5, −5	−16 ±5
35	7	111 ±2	33.9 ±1.2[4]	−23, −6, −2, +3, +4, +6, +12	− 1 ±4

[1] Initial body weight: Group I, 95–105 gm.; Group II, 195–205 gm.
[2] Deficient in riboflavin.
[3] P 0.3–0.5.
[4] P 0.1–0.2.

TABLE 3. EFFECT OF SEVERE OPERATIVE STRESS ON GALACTOFLAVIN-FED RATS ORIGINALLY WEIGHING 80 GM.[1]

Group	Blood loss, ml.	Adrenal ascorbic acid, mg.%		
		First	Second	% change
Laparotomized[2]	—	393	183	−53
	—	464	286	−38
	—	468[4]	266	−43
	—	490[5]	353	−27
Adrenal cannulation[3]	0.2	305	165	−47
	0.4	387	180	−54
	0.6	367	251	−32
	1.0	388	222	−43
	1.1	423	168	−60

[1] Range ±5 gm.; final weight 60 ±5 gm.
[2] Animals anoxic, extremities bluish, opened from sternum to pelvis; on high carbohydrate diet deficient in riboflavin 18 days.
[3] Right adrenal removed first, left adrenal cannulated then removed one hour later, body cavity remained open on each side; on high carbohydate diet deficient in riboflavin 12 days.
[4] Artificial respiration.
[5] Considerable blood loss.

pelvis along the midline, a cut made through each side to about the kidney, and one adrenal removed. In some instances, the contralateral adrenal vein was cannulated for the collection of blood. The viscera were moved to one side and covered with a saline-soaked sponge after which the animal was warmed with an electric lamp. One hour later the second adrenal was removed. The results of these procedures on the AAA are shown in Table 3.

Influence of dietary intake on the AAA response to stress. A series of experiments was performed to examine the effect of food restriction on the response to surgical stress in animals fed complete diets (i.e., Purina laboratory chow, and high carbohydrate or high protein diets supplemented with riboflavin). The complete diets were fed to young rats (80 gm.) for 13 to 16 days in amounts sufficient to maintain their weight within 3 gm. of that of similar animals fed the galactoflavin-containing, high carbohydrate or high protein diet. Table 4 reveals that AAA response to unilateral adrenalectomy was almost completely blocked in all groups of animals. Although the adrenal weights were not significantly different, the thymus glands were consistently higher in the galactoflavin-fed rats than in their pair-weighed controls.

Reversal of the inanition-induced inhibition of the AAA response to stress.

TABLE 4. INFLUENCE OF HIGH CARBOHYDRATE OR HIGH PROTEIN DIET CONTAINING GALACTOFLAVIN ON THYMUS AND TESTES WEIGHTS AND AAA RESPONSE TO STRESS IN 80 GM. RATS[1]

Group	% change in AAA		Thymus mg. ±S.E.	Testes mg. ±S.E.
	Individual values	Ave. ±S.E.		
A[2]				
Pair-weighed controls Laboratory Chow	−39, −14, −12, −10, −4, −3, −1, −1, +2, +3	− 8±4	27.5± 4.6	
Galactoflavin High carbohydrate	−37, −37, −29, −21, −18, −14, −13, −12, −11, +9	−18±4	54.4± 8.0	
B[3]				
Pair-weighed controls High carbohydrate	−6, −6, −3, +2, +13	0±4	40.7± 3.5	
Galactoflavin High carbohydrate	−16, −4, 0, 0, +17	− 1±5	72.8±11.9	
C[4]				
Pair-weighed controls High protein	−23, −19, −15, −13, −11, −7, −4, +4, +5, +6, +8, +10	− 5±3	28.2± 3.5	471±56
Galactoflavin High protein	−26, −17, −9, −5, −5, −5, −5, −3, +5, +7, +8	− 5±3	36.4± 7.4	246±29

[1] Weight range 75–85 gm.; galactoflavin diets deficient in riboflavin.
[2] High carbohydrate plus galactoflavin and pair-weighed controls on Purina laboratory chow for 13–16 days.
[3] High carbohydrate plus galactoflavin and pair-weighed controls with riboflavin (0.1 mg./gm., replacing galactoflavin) for 13 days.
[4] High protein plus galactoflavin and pair-weighed controls with riboflavin (0.4 mg./gm., replacing galactoflavin) for 15–16 days.

TABLE 5. GALACTOFLAVIN AND INANITION INHIBITION OF ADRENAL ASCORBIC ACID RESPONSE REVERSAL BY AD LIBITUM FEEDING[1]

	% change in AAA		Thymus mg. ±S.E.	Change in animal weight gm. ±S.E.
	Individual values	Ave. ±S.E.		
Group I[2] Galactoflavin-fed	−45, −39, −36, −26, +16	−26±11	69± 4	17±0.7
Group II[3] Pair-weighed	−39, −31, −28, −15, −6	−24± 6	69±16	24±0.6

[1] Initial animal weight 80±5 gm.; high carbohydrate diets.
[2] Fed galactoflavin, riboflavin-deficient diet 14 days; fed complete diet *ad libitum* 2 days.
[3] Fed complete diet restricted to quantity consumed by galactoflavin-fed, weight-matched twin; fed riboflavin-deficient diet *ad libitum* 3 days.

The results shown in Table 4 suggested that riboflavin deficiency was not the principal factor responsible for inhibition of AAA response to stress in animals on a restricted diet. This conclusion was supported by experiments in which attempts were made to overcome this block by oral or parenteral administration of riboflavin to young rats (80 gm.) fed the galactoflavin-containing, high carbohydrate diet for 2 weeks. Intraperitoneal injection of as much as 1.5 mg. of riboflavin over a 3 hour period was essentially without effect on the adrenal response. Although feeding *ad libitum* a high carbohydrate diet containing riboflavin for 2 days reversed the inhibition in rats previously given galactoflavin (Table 5), a similar effect was obtained in animals, previously pair-fed the complete diet, by feeding *ad libitum* a riboflavin-deficient diet. Thus, adequate food intake appeared to be more important than riboflavin in the re-establishment of adrenal sensitivity.

Influence of riboflavin deficiency and inanition in pair-weighed control rats on the AAA response to the stress of adrenalectomy. Eighteen weanling male rats in pairs were maintained on the high carbohydrate riboflavin-deficient diet with one of each pair receiving 0.1 mg. riboflavin per gm. of diet. The food of the rats receiving riboflavin was restricted so as to maintain their weight the same as the paired riboflavin-deficient rats. After 81 or 82 days on the diets the rats were tested for their response to stress; they weighed on the average 72 gm. The per cent AAA decrease of the second adrenal was the same for both groups (9 control rats 33±6%, S.E.; 9 riboflavin-deficient rats 32±7%, S.E.).

DISCUSSION

Forker and Morgan (1) demonstrated that the AAA response to stress was blocked in rats maintained on a riboflavin-deficient diet for 11 weeks from the time of weaning. In the present studies, inhibition of the adrenal response to unilateral adrenalectomy was produced in 80 gm. rats within 10 days by feeding riboflavin-deficient synthetic diets containing galactoflavin. These animals lost 20% of their body weight in this time, but other-

wise exhibited no specific indications of riboflavin deficiency. After 33 days there was some evidence of riboflavin deficiency, since porphyrins appeared on the nose; however, no animals died.

Older animals weighing initially 100 gm. and 200 gm. on the galactoflavin-containing diet became non-responsive to stress after 22 and 33 days respectively. At the end of this period, the body weight loss was about 40%. These results suggested that body stores of riboflavin, some other factor, or factors must be depleted before the AAA response to stress was blocked.

It was noticed that animals on the high carbohydrate galactoflavin-containing diet showed a great loss of weight in the first two weeks. This suggested that the effect of riboflavin deficiency on the AAA response might be mediated via a deficiency in nutritional factor other than this vitamin. To test this theory, pair-weighed animals on control diets were tested for their AAA response. The controls were blocked to the same extent as the fed animals, on both high carbohydrate and high protein diets, but there was evidence from the thymus gland weights that more adrenal cortical secretion was produced in the control animals under basal conditions. The control animals received sufficient riboflavin, unless food restriction caused an increased demand for riboflavin.

In the studies of Forker and Morgan (1), the AAA response of animals fed riboflavin-deficient diets was blocked to anoxic stress whereas this response was not blocked in pair-weighed control animals. These animals were on the diet for 11 weeks and previous to testing for AAA release both groups of rats were starved for 16 hours. In the present work, similar diets were employed, but the stress of unilateral adrenalectomy was used and the rats were not fasted prior to application of the stress. No prevention of the AAA response was observed. The difference in the apparent contradictory results may be due to the fact that anoxia is a weaker stress than unilateral adrenalectomy or to the superimposition of inanition on riboflavin deficiency. It is also possible that an increased demand may occur for a known substance, perhaps another vitamin. Recent studies in this laboratory (7) have shown that rats on a high carbohydrate diet restricted to 1 gm. per day show a progressively decreasing AAA response to unilateral adrenalectomy from 43% on the 5th day down to 27% on the 8th day after starting the diet. When riboflavin-deficient diets are substituted for complete diets,[4] there is less decrease in response to the stress each day, dropping from 29% on the 5th day to 8% on the 8th day. These data could indicate that the AAA response to unilateral adrenalectomy is dependent on a dietary factor or factors in addition to riboflavin. When the supply of unknown factor is limited, then the effect of riboflavin deficiency on the AAA response becomes apparent. Burch *et al.* (8) have

[4] Unpublished data.

shown that the apoenzyme of at least two enzymes, as well as the flavin-mononucleotide and flavin-adenine-dinucleotide, decrease in riboflavin deficiency. The use of the high protein control diet would seem to eliminate amino acids as a rate-limiting factor either for synthesis of ACTH or of apoenzymes. Still, the data are consistent with the hypothesis that AAA response to stress is dependent on dietary factors not yet identified. That such unknown compounds may exist has been suggested in studies of dietary stress (9, 10).

Corticotropin caused AAA depletion in the galactoflavin-fed rat blocked to the stress of adrenalectomy[4] and in the riboflavin-deficient animals blocked to the stress of anoxia (1). These animals exhibited hypertrophy of the remaining adrenal after unilateral adrenalectomy.[4] Therefore, the impairment of AAA release would appear to be located in the pituitary gland or in the brain. An even more severe stress, such as extensive laparotomy, resulted uniformly in AAA depletion. It cannot yet be ascertained whether the response to severe stress is mediated peripherally via the release of epinephrine or some other humoral agent, or centrally via the hypothalamus-pituitary portal system. However, it would seem more likely that the latter is the case and, as a corollary, that inanition impaired the central response to stress.

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ADRENAL CORTICAL SECRETION BY THE GOLDEN HAMSTER[1,2]

WILLIAM J. SCHINDLER[3,4] AND KARL M. KNIGGE[5]

Department of Anatomy, School of Medicine, University of California, Los Angeles, Los Angeles 24, California

ABSTRACT

A study of hamster adrenal venous blood as collected by cannulation from the left adrenal has shown that this species secretes hydrocortisone (Cpd. F) and no detectable "trace" steroids. The rate of blood flow through the adrenal, calculated on a body weight basis, is comparable to that of other species; however, the steroid concentration of this blood is 4–10 times less than that reported for other species. A calculated secretion rate of 0.5 mg. F/kg./24 hr./animal in the hamster is 3–10 times smaller than secretion rates of the monkey, dog, cat, ox, ferret and rabbit, and 16–48 times smaller than that of the rat. This is the lowest secretion rate of corticoids of any animal reported in the literature.

INTRODUCTION

THE classical studies of Vogt in 1943 (1) demonstrated that adrenal venous blood from various species contained adrenal cortical activity (as measured biologically) in far greater amounts than could be extracted from the glands themselves. Subsequent developments in methods of steroid chemistry have allowed later workers to be more precise concerning the actual chemical nature of the adrenal secretory products.

In 1950, Reich, Nelson and Zaffaroni (2) reported the isolation of hydrocortisone (Cpd. F) from the adrenal venous blood of dogs after the administration of ACTH. Subsequently, Bush (3, 4, 5) reported upon the qualitative and quantitative secretion rates of the adrenals of the dog, monkey, cat, sheep, ox, ferret, rabbit and rat. Trace amounts of corticosteroids such as 11-dehydrocorticosterone (Cpd. A), cortisone (Cpd. E), 11-desoxycorticosterone (DOC), aldosterone, and 17-hydroxy-11-desoxy-

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[3] Trainee Interdisciplinary Mental Health Program, University of California, Los Angeles.

[4] Present address: Department of Physiology, Baylor School of Medicine, Houston, Texas.

[5] Present address: Department of Anatomy, University of Cincinnati, School of Medicine.

corticosterone (Subst. S) have been isolated from the adrenal effluent of various species; however, Cpd. F and/or corticosterone (Cpd. B) account for 75–100% of the total corticosteroid produced in all species studied (5, 6).

No studies to date have reported data concerning adrenal cortical secretion by the golden hamster (*Mesocricetus auratus*), a species whose adrenal physiology and histochemistry differ in several respects from that of other laboratory animals. Among these differences are a reversal in the usual sexual dimorphism of the size of the adrenals (7, 8, 9, 10) and uniqueness in electrolyte balance and survival after adrenalectomy (11, 12, 13, 14).

Histologically, the hamster adrenal cortex is similar in many respects to the glands of other species (7, 9, 15, 16, 17, 18). There are, however, a number of notable cytological and histochemical differences. Although the mitochondria and Golgi apparatus of the zona fasciculata cells conform to the general descriptions of these organelles, they exhibit a strikingly different response to hypophysectomy (17) from that reported to occur in the rat and other species (19). In sudan preparations the whole of the cortex may be tinted diffusely, a coloration which may be due to lipid-rich mitochondria (15, 17). The most divergent cytological feature of the hamster adrenocortical cells is the almost complete absence of cytoplasmic lipid droplets (15, 16, 17, 18). A negative histochemical test for cholesterol (Schultz reaction) is confined by chemical analysis indicating an adrenal cholesterol concentration of zero to <0.5 mg.% (15, 17, 20, 21).

The following studies were initiated in order to determine what steroid the hamster was secreting predominantly, the rate of blood flow through the adrenal under the operative conditions necessary for this measurement and at what rate the steroid was being secreted.

MATERIALS AND METHODS

As a prerequisite to reliable direct collection of adrenal venous blood, a thorough study of the anatomical relations of the adrenal vasculature was undertaken.

Eleven adult golden hamsters (6 male and 5 female) were used in this study. A 2–5% aqueous carmine solution in 10% gelatin was used to fill the arterial system and a 2% aqueous solution to trypan blue for the venous system. The dissections were performed using a binocular dissecting microscope.

Based upon this knowledge, the cannulation technique of Bush (3) was modified appropriately to permit collection of adrenal effluent blood from hamsters which were anesthetized with nembutal (5 mg./100 gm. B. W.). A detailed description of the cannulation technique has been presented elsewhere (22). Sixty-four adult golden hamsters were successfully cannulated and are included in this study. Adrenal venous blood from three to seven animals was pooled for each determination. Male hamsters were found to be easier to cannulate because of the larger size of the adrenal gland and accompanying vessels, and because they have less perirenal and abdominal fat than females.

Steroids were extracted from adrenal effluent blood using ethyl acetate according to the method of Bush (3). Redistilled solvents were used. Extracts were partitioned (3) between petroleum ether (boiling range 30°–60° C) and 70% ethyl alcohol before being chromatographed. The steroids were chromatographed in the toluene-methanol-water

(4:3:1) system of Bush (23) and the E_2B and NB_1 systems of Eberlein and Bongiovanni (24). Quantitation of the steroids on the chromatograms was achieved colorimetrically by a modification of the blue tetrazolium method of Touchstone and Hsu (25).

The chromatographic mobility of unknown steroids was compared directly with that of authentic standards. The latter were always run on the two outer limbs of the same chromatogram under identical conditions with the unknown steroids. In no case were RF values used alone as criteria for identification. All chromatograms were examined with ultraviolet light and reflex photographs made. For the formation of derivatives, all unknowns were first separated and isolated by chromatography. The steroids were located by ultraviolet reflex photography and eluted with redistilled methanol. The method as described by Zaffaroni and Burton (26) was used for acetylation. Oxidation of steroids was accomplished using sodium bismuthate (27, 28) or chromic acid (28). Sulfuric acid chromogens were prepared according to Zaffaroni (29).

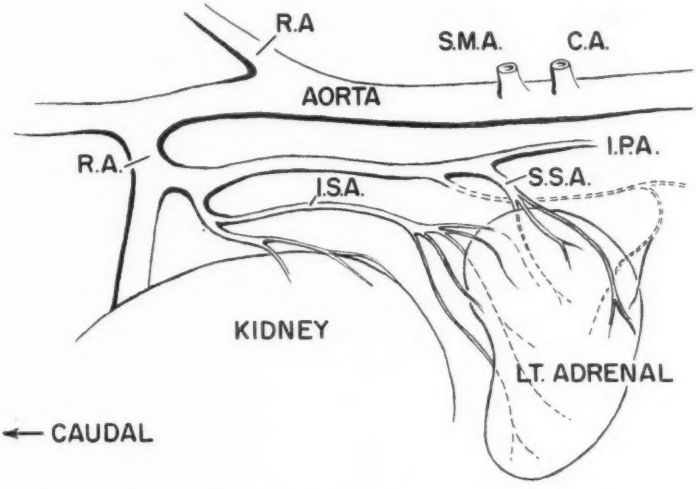
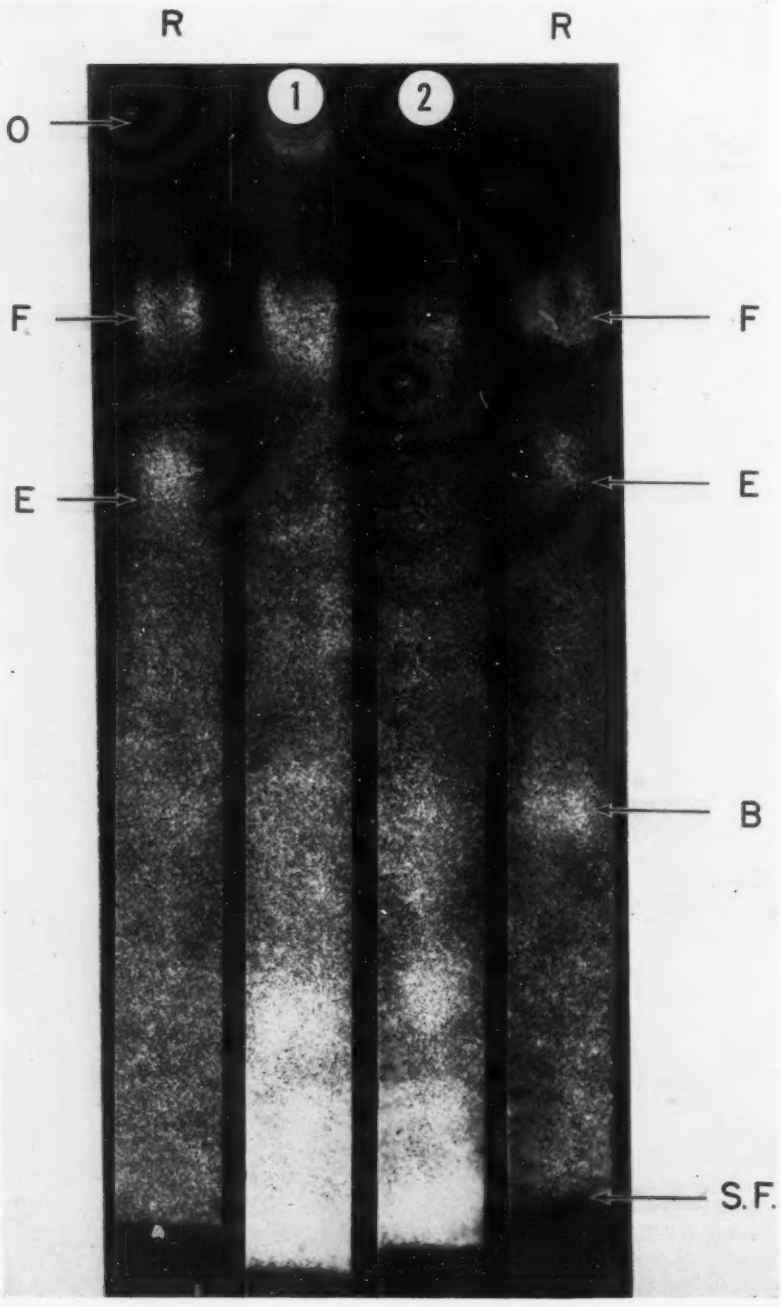


FIG. 1. Ventral view showing the predominant pattern of arterial blood supply to the left adrenal.

C.A. = Coeliac artery; I.P.A. = Inferior phrenic artery; I.S.A. = Inferior suprarenal artery; R.A. = Renal artery; S.M.A. = Superior mesenteric artery; S.S.A. = Superior suprarenal artery.

All of the arterial supply to the left adrenal came from a common stem which originated from the renal artery or from the junction of the renal artery and the aorta. This common stem divided immediately, the smaller branch contributing a small vessel to the superior pole of the kidney and a second vessel, the inferior suprarenal artery, which broke into a spray of small vessels near the adrenal and supplied its inferomedial and dorsolateral aspects. The second branch gave off a large superior suprarenal artery and a smaller branch before continuing on as the inferior phrenic artery to the diaphragm. The superior suprarenal artery also branched as it approached the adrenal so that 6–8 twigs entered to supply the medial and superomedial aspect of the gland. The smaller branch, shown in broken lines, ran deep along the dorsal body wall and gave a small branch to the superior pole of the adrenal.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RESULTS

Adrenal Blood Supply. The predominant pattern of arterial supply to the left adrenal is seen in Figure 1. The adrenal glands were closely applied to the superior pole of the kidney, enveloped by perirenal fat. In most of the animals, all of the arterial supply was derived from a common stem branching from the renal artery. Occasionally the superior suprarenal artery arose, as a common stem with the inferior phrenic artery, from the aorta. In all of the hamsters studied, a middle suprarenal artery was lacking. The anteromedial aspect of the adrenal, normally supplied by the middle suprarenal vessel, was furnished by branches from the inferior and superior suprarenal arteries.

In all animals studied, the venous drainage of the left adrenal was the same. A single major vein issued from the inferomedial surface of the adrenal gland, was joined by 2–3 extremely small veins from the perirenal fat and, coursing ventral to the arterial vessels, drained into the left renal vein. The latter was approximately 6–7 mm. in length and 2 mm. in diameter. The suprarenal vein was 3–4 mm. long and 0.4–0.5 mm. in diameter. Its point of entry into the renal vein was usually at the latter's approximate mid-point, although some variation was observed. The gonadal vessel entered the renal vein approximately 1–2 mm. from the junction of the renal with the inferior vena cava. These anatomical features of arterial supply and venous drainage permitted direct collection of effluent blood from the left adrenal gland.

Adrenal Cortical Secretion. Hydrocortisone (Compound F) has been consistently recovered as the major secretory product from the pooled adrenal venous blood of adult hamsters. No "trace" steroids were found in any of the blood samples. The identity of the steroid as Cpd. F was determined by the following series of tests. Its chromatographic mobility was identical with authentic Cpd. F in both the toluene-methanol-water and E_2B chromatography systems (Fig. 2). The steroid obtained from hamster blood would not separate from authentic Cpd. F on mixed chromatograms (Fig.

FIG. 2. Ultraviolet reflex print of a chromatogram run in Bush's toluene:methanol:water (4:3:1) system showing the Cpd. F isolated from the adrenal venous blood of hamsters.

B = Cpd. B
E = Cpd. E
F = Cpd. F
F-Ac = Cpd. F acetate
O = Origin
R = Reference limbs on which steroid standards were run.
S.F. = Solvent front
① and ② = Limbs on which adrenal venous effluent were run.

Cpd. F was the only alpha-ketolic steroid isolated. Ultraviolet absorbing material at solvent front of limbs ① and ② was non-alpha-ketolic.

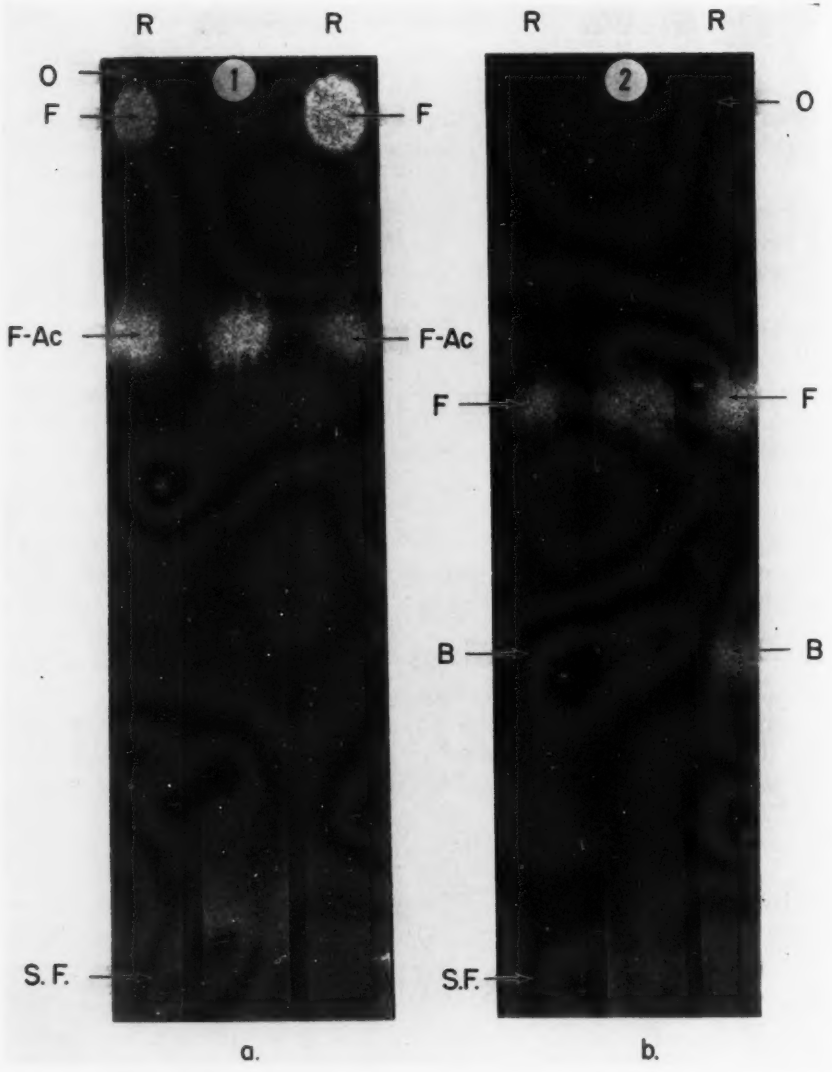

FIG. 3. Ultraviolet reflex prints of chromatograms run in the NB_1 system (Fig. 3a) and the E_2B system (Fig. 3b) showing mixed chromatograms of Cpd. F acetate (a) and free Cpd. F (b) respectively. (See Fig. 2 for key to abbreviations.)

1. The Cpd. F isolated from hamster adrenal venous effluent was eluted with methanol from limbs 1 and 2 of the chromatogram shown in Figure 2 and run as mixed chromatograms as the acetate and free alcohol.
2. The acetylated Cpd. F (Limb 1) did not separate from authentic Cpd. F acetate after running as a mixed chromatogram in the NB_1 system.
3. Cpd. F (Limb 2) did not separate from authentic Cpd. F after running as a mixed chromatogram in the E_2B system.

3). The steroid in question showed positively on ultraviolet reflex photography, thus demonstrating the probable possession of an alpha-beta unsaturation in ring A. It also stained blue when sprayed on the chromatogram with blue tetrazolium, thus indicating an alpha-ketol grouping in the molecule. The acetylated steroid had a chromatographic mobility identical to that of authentic F acetate. Further, it did not separate from F acetate on mixed chromatograms (Fig. 3). Oxidation with sodium bismuthate gave an ultraviolet absorbing compound which had the chromatographic mobility of 11β-hydroxy-androst-4-ene-3, 17-dione and which gave the characteristic positive reaction of a 17-ketosteroid when sprayed with Zimmer-

TABLE 1. SUMMARY OF THE IMPORTANT PARAMETERS OF *in vivo* HYDROCORTISONE SECRETION AND BLOOD FLOW

Group	No. animals	Sex	Mean body wt. gm.	Mean lt. adrenal wt. mg.	Mean collection time hr.	Mean blood vol. ml.	Total μg. F collected	Flow rate ml./hr./100 gm. B.W.	Secretion rate		Concentration μg. F/ml.
									μg. F/100 mg. adrenal/hr.	μg. F/adrenal/100 B.W./hr.	
#HC	1	M	150	—	1.17	2.50	—[a]	1.43	—	—	—
#HD	7	F	154	—	1.13	0.70		1.10	—	—	—
#HD-1	5	M	149	18.5	0.98	1.28	—	0.88	—	—	—
#HE	3	M	147	15.4	1.11	2.43	—	1.49	—	—	—
#HF	4	M	151	17.1	1.12	1.38	—	0.80	—	—	—
#HG	6	M	124	12.1	0.68	1.00	10	1.19	20.40	1.98	1.67
#HJ	4	M	146	13.6	0.83	1.63	13	1.32	27.64	2.55	1.92
#HK	7	M	126	12.1	0.76	1.10	15	1.15	23.31	2.22	1.95
#HL	4	M	146	17.2	0.92	1.90	8	1.41	12.62	1.49	1.05
#HM	4	M	165	14.3	0.57	1.88	5	1.98	15.24	1.32	0.67
#HN	5	M	146	14.3	0.93	2.00	20	1.48	30.16	2.96	2.50
#HO	6	M	142	13.6	0.75	1.50	15	1.40	24.48	2.34	2.22
#HP	5	M	159	16.9	1.22	2.58	20	1.33	19.45	2.06	1.55
#HQ	3	M	124	13.6	1.11	2.00	8	1.43	17.48	1.92	1.32
Mean ± Standard Deviation					*0.86 ± .20	*1.73 ± .49		1.32 ± .29	21.20 ±5.7	2.09 ± .50	1.65 ± .57

* Based on #HG to #HQ.
[a] Cpd. F from #HD to #HF used for sulfuric acid chromogens and derivative formation.

man reagent. When oxidized with chromic acid, the steroid yielded an ultraviolet absorbing compound which had the chromatographic mobility of androst-4-ene-3, 11, 17-trione and gave a positive 17-ketosteroid reaction. The steroid demonstrated an identical sulfuric acid chromogen to that of Cpd. F. The above evidence is consistent with the identification of hydrocortisone as the steroid secreted by the hamster adrenal.

The data concerning blood flow and secretion rates have been summarized in Table 1, with values reported and calculated on the basis of the pooled blood samples from 3–7 animals per group. Pooling of blood was necessary in order to get enough corticoid to measure. The range of adrenal venous effluent collected was 1.0–2.56 ml./animal with a mean of 1.73 $\pm$.49 ml./animal. All values are reported as mean $\pm$standard deviation. The duration of the collection period was 0.86 $\pm$.20 hrs./animal with a range of 0.57–1.22 hrs./animal. The mean flow rate of blood through the left adrenal was 1.32 $\pm$.29 ml./hr./100 gm. body weight. When the secretion rate of Cpd. F was calculated on an adrenal weight basis, the mean secretion rate was 21.20 $\pm$5.7 μg. F/100 mg. adrenal/hr. with a range of 12.62–30.16. A mean of 2.09 $\pm$.50 μg. F/adrenal/100 gm. body weight/hr. was

determined when the secretion rate was calculated on a body weight basis. The concentration of the hormone in the adrenal venous blood was 1.65 $\pm$.57 μg. F/ml.

DISCUSSION

The pattern of adrenal corticoid secretion as seen in all species studied, that is the predominant secretion of Cpd. F or Cpd. B or a mixture of the two (4, 6), is seen also in the hamster. The failure to identify any "trace" corticoids in hamster adrenal venous blood may be a real lack of these substances or may be due to the small amounts of blood which were analyzed. Whether the secretion of one or two predominant adrenal steroids is genetically fixed, as suggested by Bush, remains to be proven unequivocally. Nevertheless, the studies by Bush (4) and also Eik-Nes (30) in particular lend support to the theory that, under normal conditions, each species has a characteristic secretion product of the adrenal cortex which is Cpd. F, Cpd. B or a mixture of the two. These species differences in adrenal corticoid secretion cannot be correlated with diet or mode of life of the animal.

When the secretion rates of Cpd. F by hamsters are calculated so as to be compared with the figures reported by Bush (4), it is seen that hamsters secrete ca. 0.5 mg. F/kg./24 hr./animal. This value is 3–10 times smaller than the secretion rates reported for such species as the monkey, dog, cat, ox, ferret and rabbit, and 16–48 times smaller than the 8–24 mg./kg./24 hr./animal reported for rats. Employing the same chromatographic and quantitation techniques as used in this study, we observed a mean secretion rate in the rat of 19 mg./kg./24 hrs. (31). The levels of 17-hydroxycorticosteroids found in peripheral blood of hamsters are the lowest in any animal hitherto tested (30). The mean blood flow through the left adrenal of rats was 14.6–20.0 ml./adrenal/kg./hr. (32). Calculated on this same basis, hamsters have a mean flow rate of 13.2 ml./adrenal/kg./hr. Thus, it is seen that on a body weight basis, the rate of flow of blood through the left adrenal of hamsters is comparable to that of other species. The former has a mean concentration of 1.65 μg. of steroid/ml. adrenal venous blood which accounts in part for low secretion rate of steroid by the hamster adrenal. In addition, this low secretion rate may also be due to a relative lack of preformed steroid or precursor storage in the adrenal.

In view of the fact that the steroid secretion rates of all animals studied by Bush were fairly comparable, it must be concluded that hamsters are an exceptional species and have a considerably slower secretion rate of adrenal corticoid as measured in adrenal venous blood.

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IN VITRO STUDIES AND ADRENAL STEROIDOGENESIS BY THE GOLDEN HAMSTER[1,2]

WILLIAM J. SCHINDLER[3,4] AND KARL M. KNIGGE[5]

Department of Anatomy, School of Medicine, University of California, Los Angeles, Los Angeles, California

ABSTRACT

No detectable amounts of steroid hormones can be recovered by direct extraction of hamster adrenal tissue or after incubation of adrenal slices in Krebs-Ringer Bicarbonate medium. Adrenal slices or cell-free homogenate preparations can, however, readily convert various steroid precursors to hydrocortisone (Cpd. F), cortisone (Cpd. E), corticosterone (Cpd. B), dehydrocorticosterone (Cpd. A) 17-hydroxy-11-desoxycorticosterone (Subst. S) and at least three other unknown alpha ketols. It was demonstrated that hamster adrenal glands contain the 3β-ol dehydrogenease system, the C-11β, C-17 and C-21 hydroxylase enzyme systems.

Sodium acetate was not converted to adrenal corticoids either by incubation with adrenal slices or by incubation with cell-free homogenate preparations. On the other hand, sodium acetate-1-C^{14} was readily converted to radioactive Cpd. F *in vivo*, thus demonstrating that the hamster can incorporate acetate into corticoids.

A cell-free homogenate preparation with added cofactors was able to convert cholesterol-4-C^{14} to radioactive Cpds. F, E, B and A. Adrenal slices (whole cell preparations) were unable to accomplish this conversion. Likewise, it has not been possible to demonstrate the *in vivo* conversion of infused cholesterol-4-C^{14} to radioactive Cpd. F. These findings suggest that the conversion of cholesterol to corticoids depends upon the permeability to the cholesterol, i.e., the availability of the substrate to be acted upon. Evidence from these experiments suggests that cholesterol may not be a normal adrenal corticoid precursor in the hamster.

INTRODUCTION

HECHTER'S original studies (1, 2) involving the perfusion of blood through isolated beef adrenal glands and analysis of the perfusate revealed that Cpd. F and Cpd. B were the predominant steroids produced *in vitro*. Steroid substrates were perfused through this preparation and the

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[2] Presented by William J. Schindler to the Graduate School of the University of California, Los Angeles in partial fulfillment for the degree, Doctor of Philosophy in Anatomy.

[3] Trainee Interdisciplinary Mental Health Program, University of California, Los Angeles.

[4] Present address: Department of Physiology, Baylor School of Medicine, Houston, Texas.

[5] Present address: Department of Anatomy, University of Cincinnati, School of Medicine.

isolation of the products formed established that adrenal tissue possessed enzyme systems for the step-wise introduction of hydroxyl groups at C-17, C-21 and C-11 of the progesterone molecule (2, 3). The existence of these enzyme systems was confirmed and their characteristics elucidated by other workers using adrenal slice and homogenate preparations (4, 5, 6, 7).

With the assumption that cholesterol is a precursor, and that Cpds. F and B are end products of steroidogenesis, Hechter and his group postulated the type of reactions necessary to achieve this transformation. By perfusing various chosen steroid substrates through bovine adrenals and subsequent isolation of the products, the Worcester Foundation group has been able to evaluate the enzymatic capacities of adrenal tissue to effect specific reactions. The reaction sequence of corticosteroid biosynthesis was proposed in 1950 on the basis of the ability of substrates to react (2, 3, 8), and has since been considerably strengthened by the reproduction of each of the postulated conversions by adrenal homogenate preparations (8, 9, 10). In this scheme pregnenolone is the major product of cholesterol side chain scission (11) with progesterone the "key intermediary" arising from pregnenolone, and being converted by separate pathways to Cpds. F and B (2, 8).

The relative absence of cholesterol and other lipids from the hamster adrenal (see references in previous paper; 12) creates a focal point of considerable interest with respect to steroidogenesis by the adrenals in this species as well as a possible significance to adrenal physiology in general. Studies to date on hamster adrenals have been restricted mainly to gross morphological, histological and histochemical features. These studies have adequately presented the differences exhibited by the hamster adrenal; however, there is a considerable gap in our understanding of basic adrenocortical function in this species. There is no knowledge of the enzymatic pathways of steroidogenesis, or the rates of synthesis and secretion of the products. This study was undertaken to begin the investigation of some of the fundamental aspects of *in vitro* adrenal steroidogenesis.

METHODS

Adrenal Slices in Vitro. Hamsters were stunned by a cervical blow, adrenals removed immediately and placed in iced petri dishes. After fat and adhering connective tissue were removed from the glands, they were halved and distributed evenly so that each flask would contain the same amount of tissue from each animal. The pooled adrenal halves were weighed (40 to 100 mg. per incubation flask) and placed in Warburg flasks containing 2 ml. of Krebs-Ringer-Bicarbonate media (200 mg.% glucose added) of pH 7.4. Steroid substrates, dissolved in propylene glycol, were added to the medium in amounts of 0.2 or 0.4 mg. steroid/100 mg. adrenals. After prior gassing (95% O_2–5% CO_2) for 10 minutes, the flasks were incubated for 2 to 3 hours at 37° C with continuous shaking. After incubation, the medium was removed, extracted three times with three separate volumes of methylene chloride, partitioned and chromatographed as described previously (12). After photography in the ultraviolet, dried chromatograms were sprayed with blue tetrazolium for quantitation (12).

Cell-Free Adrenal Homogenates. Pooled adrenal glands (ca. 320 mg. per experiment)

were homogenized in enough iced 0.25 *M* sucrose to make a 10% homogenate, which was centrifuged for 10 minutes at 5° C at a speed of 1900 RPM (700×g). The sucrose supernatant was removed and divided equally into four Warburg flasks. To each flask was added 1.5 ml. Krebs-Ringer-Bicarbonate, pH 7.4 (200 mg.% glucose), and "standard cofactor additions." The latter consisted of 300 mg.% nicotinamide, 0.06 *M* sodium fumarate, ATP and DPN both at 0.2% of the adrenal weight, (i.e., 0.2 mg. of each per 100 mg. adrenal tissue). Each of the standard additions was made up separately in double distilled water and added to the incubation flask in a volume of 0.1 to 0.2 ml. Steroid substrates were added as described for the adrenal slices. The flasks were gassed for 10 minutes, followed by 3 hours of incubation at 37° C. The medium was then extracted with chloroform, partitioned between water and petroleum ether, and the aqueous phase subsequently extracted with methylene chloride as modified from Eik-Nes (13).

C^{14}-Labeled Cholesterol and Acetate in Homogenates. Cell-free homogenates were prepared as described above. Cholesterol-4-C^{14} (Nuclear Chicago; specific activity 2 5 microcuries/mg.) was dissolved in propylene glycol and 0.23 ml. of this labeled cholesterol (1.15 microcuries) was incubated in each flask. Sodium acetate-1-C^{14} (Nuclear Chicago; specific activity 2.2 millicuries/millimole) was dissolved in double distilled water at a concentration of 6.8 mg. acetate/ml. water. For each incubation, 0.2 ml. of this solution were used (10 micromoles acetate equal to 2.2 microcuries).

After chromatography, the steroids produced by incubation were located by ultraviolet reflex photography and marked on the chromatographic strip. The entire length of the strip was cut consecutively into segments of ca. 1.8 cm. each. Care was taken to insure that the area of each separate steroid was confined to one or at most two segments. Each segment of chromatogram was placed on a separate planchet, and its radioactivity determined with a thin window Nuclear Chicago D47 flow gas counter. The areas which contained steroid and radioactivity were then eluted with methanol, the steroids acetylated, and chromatographed in the NB_1 system. Following location of the spot with ultraviolet reflex photography, the strip was cut as before to determine the radioactivity of the acetylated compound. The cut strips were then sprayed with blue tetrazolium for quantitation.

C^{14}-Labeled Acetate and Cholesterol in Vivo. The left adrenal glands of four male hamsters were cannulated and adrenal effluent blood collected. When blood began to flow through the cannula, each animal was injected via the femoral vein with 10 microcuries of sodium acetate-1-C^{14} (specific activity 1 millicurie per millimole). Similarily, 10 microcuries of cholesterol-4-C^{14} (specific activity 19.35 millicuries per millimole) were injected after cannulation of five male hamsters. The labeled cholesterol was dissolved in a small amount of absolute alcohol and saline added to make a 10% alcoholic suspension. Adrenal venous blood from each group was pooled, extracted, partitioned between petroleum ether and 70% ethanol and chromatographed as described previously. The Cpd. F isolated from these blood samples was located on each chromatogram by ultraviolet reflex photography. This area of each chromatographic strip was cut out and eluted with methanol, then plated out on a glass coverslip. The preceding and each succeeding 3–3.5 cm. length of the chromatogram was cut out also, eluted with methanol and plated out on glass coverslips. The radioactivity of each area was measured in a windowless gas flow counter.

RESULTS

Stored Hormones. No steroids possessing an alpha-ketol grouping in ring D or an alpha-beta unsaturation in ring A have been recovered from batches of hamster adrenal glands after extraction with methylene chloride,

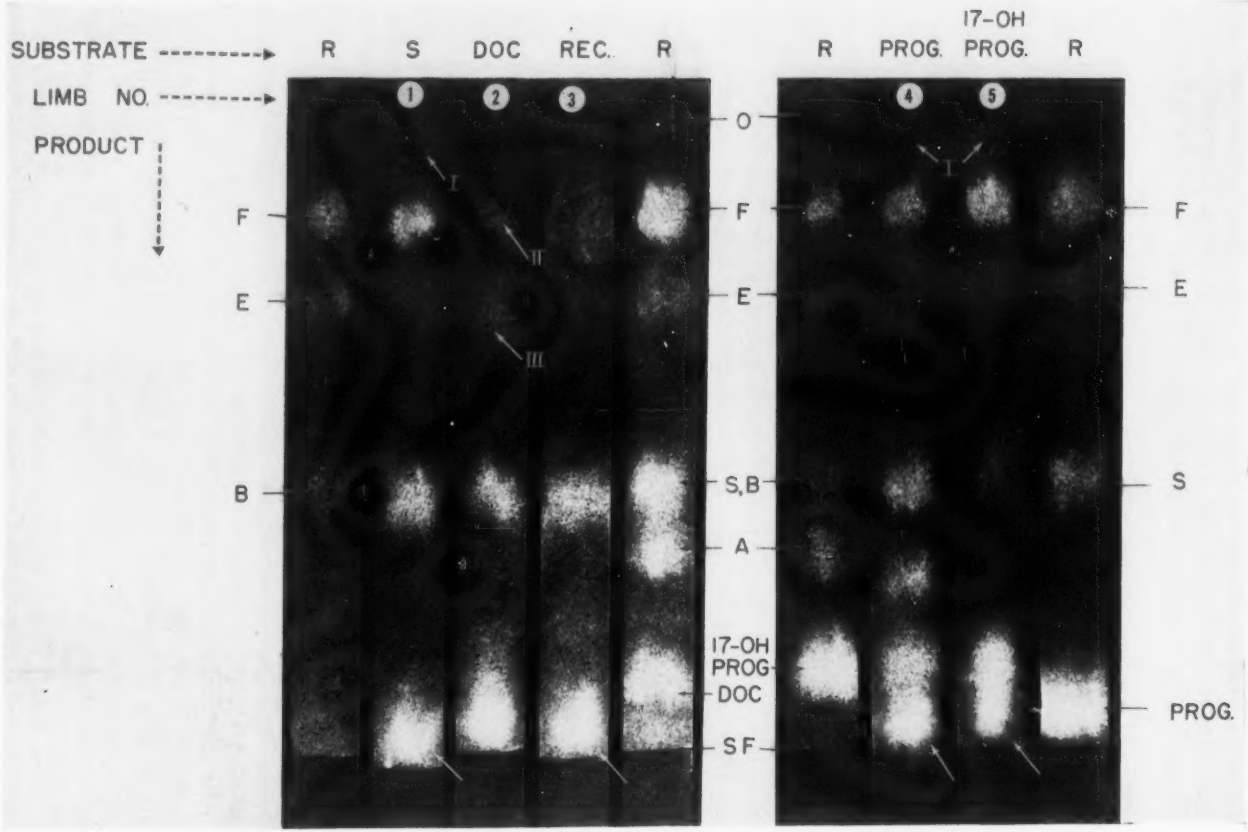
chloroform or ethyl acetate. Numerous attempts to extract adrenal steroids from the pooled glands of 3 to 20 hamsters (50 to over 400 mg.) by homogenizing the glands in Krebs medium followed by extraction of this aqueous medium with the above-mentioned solvents or by direct solvent extraction of the glands have proven fruitless. Since amounts of steroids of approximately 1–0.8 μg. are readily detected by the methods employed (U.V. photography and blue tetrazolium spraying), and since our recovery studies indicate an approximate 80% efficiency in the overall process of extraction, partitioning and chromatography, failure to isolate any hormone from a 400 mg. batch of hamster adrenals would indicate a storage of hormone, if any, of less than 0.25 μg./100 mg. adrenal. This amount would be 10 times less than the lowest reported stored hormone concentration in other species (guinea pig; 14).

Adrenal Slices in Vitro. Without the addition of any steroid substrates, hamster adrenal tissue (100–246 mg. of adrenal slices per flask) has, in five separate experiments, failed to produce any steroids when incubated for 2½–3 hours at 37° C in Krebs-Ringer-Bicarbonate medium (pH 7.4) or in heparinized hamster blood. In control experiments run at the same time, rat adrenal slice incubates produced 60–100 μg. of alpha-ketolic steroids (Cpds. F and B, aldosterone, and two unknowns) from endogenous substrates.

Using these conditions of incubation under which no corticoids were produced from endogenous sources by hamster adrenals, steroid substrates were added to the Krebs medium in an attempt to determine the ability of the adrenal enzyme systems to convert these substrates to the final products (adrenal corticoids) in 2 or 3 hours of incubation. The choice of substrates used was influenced primarily by the scheme of corticosteroid biosynthesis proposed by Hechter *et al.* (2, 3). The qualitative results of 92 separate *in vitro* incubations using 16 different steroid substrates are summarized in Table 1 and Figures 1 and 2. No alpha-ketolic steroids were recovered when acetate, acetate plus ACTH, squalene, squalene plus ACTH, cholesterol, cholesterol plus ACTH, or cholestan-3-one was added to the Krebs medium (0.1–0.4 mg. steroid substrate/100 mg. adrenal) and incubated with adrenal slices for 3 hours at 37° C.

In identical experiments, steroid substrates such as pregnenolone, 17-hydroxypregnenolone, progesterone, desoxycorticosterone (DOC) and Subst. S were converted to alpha-ketolic steroids. Incubations with pregnenolone as substrate produced Cpds. F, E, B and one unidentified alpha-ketol more polar than Cpd. F[6] (Unknown No. 1, Table 1). In order to form

[6] The identity of each of the α-Ketols produced was determined primarily by its chromatographic mobility, running in parallel with authentic steroid standards in at least two systems, and its reactions to ultraviolet light and blue tetrazolium. When sufficient amounts of α-Ketols were recovered, samples were identified further by acetate derivative formation, mixed chromatograms or sulfuric acid chromogens.


SUBSTRATE
R
S
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PROG.
17-OH PROG.
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LIMB NO.
1
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PRODUCT
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these incubation products, the pregnenolone molecule must be oxidized in ring A and hydroxylated at C-11, C-17 and C-21. These reactions indicate the presence of the following enzyme systems: the 3-β-ol dehydrogenase (the ring A oxidizing system), the 11-β hydroxylase, the 17-hydroxylase and the 21-hydroxylase. Both 17-hydroxypregnenolone and 17-hydroxyprogesterone were converted to Cpds. F, E and Subst. S. Progesterone, the "key" compound in Hechter's reaction sequence was oxidized at positions 11, 17 and 21 forming Cpds. F, E, B, A and traces of Unknown No. 1 and two other unidentified alpha-ketolic steroids (Unknowns No. II and No. III; Table 1).

Incubation of DOC with hamster adrenal slices produced Cpds. B and A and significant amounts of Uknowns No. II and No. III, which had the chromatographic mobility of Cpd. F and Cpd. E respectively in Bush's toluene-methanol-water (4:3:1) system (15; Fig. 1). These two com-

FIG. 1. Ultraviolet reflex prints of chromatograms run in Bush's toluene-methanol-water (4:3:1) system (15) showing the steroids produced from various substrates incubated with hamster adrenal slices.

A = Cpd. A
B = Cpd. B
DOC = Desoxycorticosterone
E = Cpd. E
F = Cpd. F
O = Origin
PROG. = Progesterone
17 OH PROG. = 17 Hydroxyprogesterone
R = Reference limbs on which steroid standards were run
S.F. = Solvent front

1. Unknown Steroids No. I–III are indicated by arrows and accompanying numbers on the chromatogram. All three are alpha-ketolic steroids.
2. Incubations with Substance S as substrate (Limb 1) led to the recovery of Cpd. F, traces of Cpd. E, Unknown Steroid No. I and unconverted Substance S. The ultraviolet (U.V.) absorbing spot at the solvent front (arrow) did not react with blue tetrazolium and was not an alpha-ketol.
3. Unknown Steroids No. II and No. III had the same chromatographic mobility as Cpds. F and E respectively in this system, and were recovered after incubation with DOC as substrate (Limb 2). Cpd. B was also produced.
4. Known quantities of Cpd. F and Subst. S were incubated with boiled adrenals and the media was treated identically with the unknowns to test the percent recovery (Rec., Limb 3).
5. Incubations with progesterone as substrate (Limb 4) produced Cpds. F, E, B, A and Unknown No. I. The U.V. spot running with the solvent front of this limb (arrow) was not an alpha-ketol, and was probably unconverted progesterone. The U.V. absorbing spot slightly above this was also non-alpha-ketolic and may have been 17-hydroxyprogesterone.
6. Incubations with 17-hydroxyprogesterone as substrate (Limb 5) produced Cpds. F and E, Subst. S and Unknown No. I. The U.V. absorbing spot (arrow at solvent front) was non-alpha-ketolic, and was probably unconverted 17-hydroxyprogesterone.
7. Unknown Steroid No. I was more polar than Cpd. F and was produced by incubations with Subst. S (Limb 1), progesterone (Limb 4) and 17-hydroxyprogesterone (Limb 5).
8. Subst. S and Cpd. B have the same chromatographic mobility in this system (reference limbs).

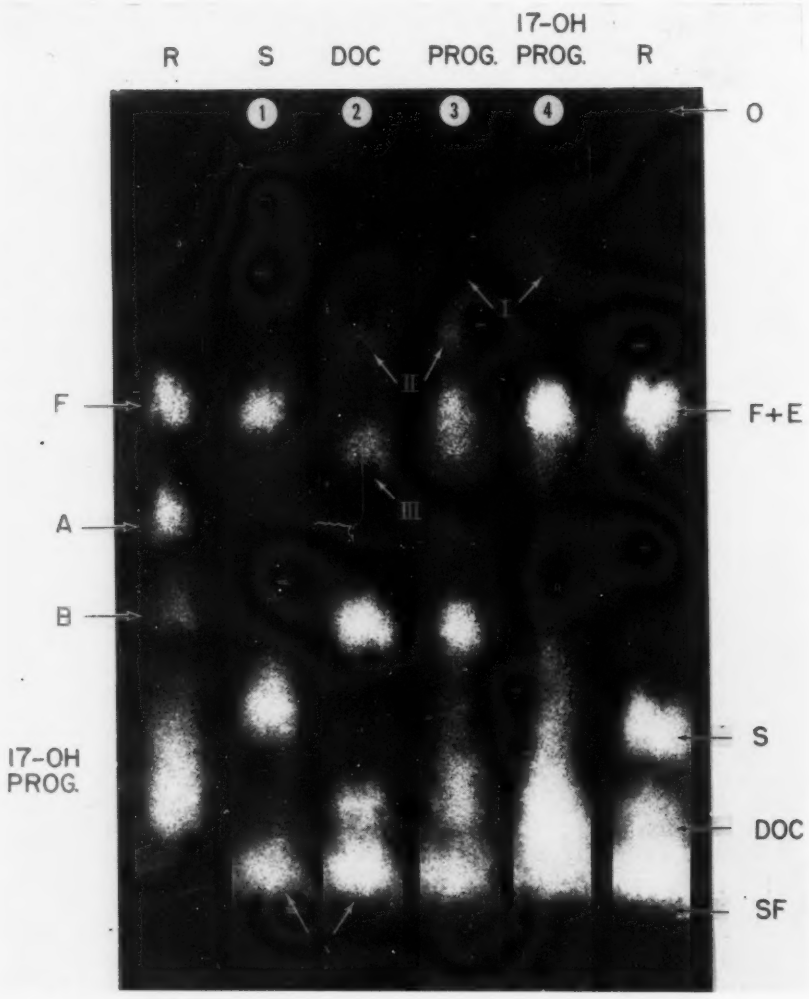

FIG. 2. Ultraviolet reflex print of a chromatogram run in the E_2B system (16) showing the steroids produced from various substrates incubated with hamster adrenal slices. (see Fig. 1 for key to abbreviations.)

1. Incubations with Subst. S as substrate (Limb 1) produced Cpd. F. Unconverted Subst. S was also recovered.
2. Incubations with DOC as substrate (Limb 2) produced No. II, No. III and Cpd. B. No. II is more polar then Cpd. F and No. III is less polar than Cpd. E in this chromatography system. Neither Cpd. F nor Cpd. E appeared to be produced from DOC incubations. Unconverted DOC was seen near the solvent front.
3. Incubations with progesterone as substrate (Limb 3) produced Cpd. F and Cpd. E

→

TABLE 1. ALPHA-KETOLIC STEROIDS PRODUCED BY INCUBATION OF VARIOUS STEROID PRECURSORS WITH HAMSTER ADRENAL SLICES AND THE ENZYME SYSTEMS DEMONSTRATED BY THESE CONVERSIONS

Steroid substrate supplied	No. hamsters	No. determinations	Alpha-Ketolic steroid produced					Enzyme system demonstrated			
			Cpd. F	Cpd. E	Cpd. B	Cpd. A	Other	3β-ol Dehydrogenase	11β Hydroxylase	17α Hydroxylase	21 Hydroxylase
None	39	5	—	—	None	—	—				
Acetate	20	2	—	—	None	—	—				
Squalene	14	4	—	—	None	—	—				
Cholesterol	57	11	—	—	None	—	—				
Cholestan-3-one	10	1	—	—	None	—	—				
Pregnenolone	30	7	+	+	+		No. I	+	+	+	+
17-OH Pregnenolone	12	3	+	+	−	−	Subst. S	+	+		+
Progesterone	40	8	+	+	+	+	No. I, II, III		+	+	+
11β-OH Progesterone	12	3	+*	+*	+					?	+
17-OH Progesterone	24	7	+	+	−	−	No. I, Subst. S		+		+
DOC	44	10			+	+	No. II, III		+		
Subst. S	62	24	+	Trace	−	−	Unconverted Subst. S; No. I		+		
Cpd. F	7	2	+	−	−	−	−				
Cpd. B	4	1	−	−	+	−					
Cpd. E	4	1	−	+	−	−					
Cpd. A	4	1	−	−	−	+					
Δ^4 Cholesten-3-one	10	1	Trace	+	+	+	−		+	+	+

* Not definitely characterized other than chromatographic mobility and reactive groups. No. I = unidentified Cpd more polar than F.

No. II and III = Unidentified Cpds. which travel with F and E respectively in Bush's (15) toluene-methanol-water system but which separate from them in the E_2B system (16) and after acetylation and chromatography in the NB_1 system (16).

pounds, when chromatographed in Eberlein and Bongiovanni's (16) E_2B system, however, proved to be respectively more polar than Cpd. F (Rf approximately 0.28) and less polar than Cpd. E (Rf 0.43; Fig. 2). Acetylation of these unknowns and subsequent chromatography of their acetates in the NB_1 system (16) showed that Unknown No. II separated from Cpd. F acetate and, with an Rf value of approximately 0.58 in the NB_1 system, migrated between Cpd. E acetate and Cpd. B acetate, while the acetate of Unknown No. III separated from Cpd. E acetate and had a mobility (Rf ca. 0.86) even faster than Cpd. A acetate. Substance S was hydroxylated at C-11 forming Cpd. F and occasionally traces of Cpd. E were also recovered. When used as substrates, neither Cpds. F, B, E nor A were acted upon by adrenal enzyme systems, and only the original compounds were recovered after the incubation (Table 1). Incubation with 11β-hydroxyprogesterone as substrate produced predominantly Cpd. B and also two compounds which had the chromatographic mobility of Cpds. F and E but which were

(these two steroids do not separate in this system), Cpd. B, a trace of Cpd. A and traces of two alpha-ketols more polar than Cpd. F (No. I and No. II). The trace of No. III produced did not separate from Cpd. F plus E. the U.V. absorbing spots at the solvent front and slightly above are probably progesterone and 17-hydroxyprogesterone respectively—neither of which is an alpha-ketol.

4. Incubations with 17-hydroxyprogesterone as substrate (Limb 4) produced No. I, Cpds. F plus E and Subst. S. The U.V. absorbing material running near the solvent front is non-alpha-ketolic and is probably unconverted 17-hydroxyprogesterone.
5. The U.V. absorbing spots at the solvent fronts of Limbs 1 and 2 (arrows) were lipid-like materials which were not alpha-ketols.

TABLE 2. QUANTITATIVE DATA ON THE AMOUNTS OF ALPHA-KETOLIC STEROIDS PRODUCED BY INCUBATION OF HAMSTER ADRENAL SLICES WITH VARIOUS STEROID PRECURSORS ADDED AS SUBSTRATES

Steroid substances supplied	Substrate ave. amt. μg.	No. flasks	Ave. adrenal wt. mg.	Ave. incubation time hrs.	Ave. μg. of major steroids produced					Ave. % of original substrate converted to these cpds.				% of recovered steroid converted to these cpds.		p values
					F	E	B	Other	Total	F	E	B	Total	F+E	B	
Pregnenolone	322	4	80.5	3	1.63	3.37	6.65	3.1	14.75	0.50	1.04	2.05	4.58	35.25 ± 7.4[d]	47.55 ± 13.4	.229
Progesterone	306	6	77.1	3	18.78	4.56	15.22	2.95	41.51	6.14	1.49	4.97	13.57	55.25 ± 19.0	37.36 ± 15.0	.102
17-OH Progesterone	303.3	6	75.4	3	23.20	4.04	12.90[a]	1.75	41.89	7.65	1.33	4.25[a]	13.81[a] (29.61)[c]	69.44 ± 18.4	27.70 ± 16.55[a]	.003
DOC	115	4	55.5	2	4.50[b]	6.58[b]	21.98	3.9	37.96	3.91[b]	6.59[b]	19.11	33.01 (16.09)[c]	33.14 ± 6.46[b]	58.73 ± 5.35	<.001
Subst. S	78	4	38.9	2	12.55		22.0[a]		34.55	16.09		28.21[a]	44.29 (9.72)[c]	35.74 ± 6.05	64.28 ± 8.25[a]	.001
Subst. S	100	6	42.0	2.58	9.72		9.22[a]		18.93	9.72		9.22[a]	18.93 (19.00)[c]	52.30 ± 8.3	47.70 ± 8.3[a]	.360
Subst. S	119.3	6	71.55	2.67	22.25		5.58[a]	trace	28.25	18.65		4.68[a]	23.68	78.63 ± 5.8	20.23 ± 7.4[a]	<.001

[a] These values refer to Subst. S.
[b] These values refer to Unknowns No. II and No. III.
[c] Figures for total per cent recovery when the unconverted DOC and Subst. S are not included in the total.
[d] Values are reported as mean ± standard deviation.

TABLE 3. ALPHA-KETOLIC CORTICOIDS PRODUCED BY INCUBATION OF VARIOUS SUBSTRATES WITH A CELL-FREE ADRENAL HOMOGENATE PREPARATION[a]—QUALITATIVE AND QUANTITATIVE DATA

Steroid substrate supplied	No. hamsters	No. determinations	Alpha-Ketolic steroids produced					Ave. μg. of major steroids produced					Net μg. produced from substrate and significance			
			Cpd. F	Cpd. E	Cpd. B	Cpd. A	Other	F	E	B	A	Other	μg. E	p	μg. A	p
None (Endogenous)	11	3	–	+	–	+	–	–	2.6 ±1.4[b]	—	0.67±0.76	—				
Acetate	28	8	–	+	–	+	–	—	3.4 ±1.24	—	<1	—	0.8		—	
Squalene	4	1	–	+	–	–	–	—	7.0	—	7.7	—	4.4		7.03	
Cholesterol	28	8	Occasional Trace	+	Occasional Trace	+	No. I	1.72	6.44±1.9	1.26	3.31±1.2	No. I 0.75	3.84	.011	2.64	.006
Cholestan-3-one	3	1	–	+	–	+	–	—	5.1	—	2.5	—	2.5		1.83	

[a] Cell-free homogenate in 0.25 M sucrose
1.2–1.4 ml. Krebs-Ringer Bicarbonate (pH 7.4) (200 mg.% glucose)
300 mg.% nicotinamide
.06 M fumarate
ATP } .2% of adrenal weight
DPN } i.e., 200 μg./100 mg. adrenal
} "Standard cofactor additions" added separately in volumes of 0.1–0.2 ml.
[b] Values are reported as mean ± standard deviation.
No. I = Unidentified steroid more polar than Cpd. F.

produced in amounts too small to allow further confirmatory characterization.

Quantitative data of the more important incubations are summarized in Table 2. The amount of the original substrate converted to alpha-ketolic steroids ranges from 4.58% for pregnenolone to 29.61% for DOC. The similarity of the percent of progesterone and 17-hydroxyprogesterone which was converted to alpha-ketols (13.57% and 13.81% respectively; Table 2) is noticeable. Progesterone can readily be converted to either Cpd. F or Cpd. B, with only a slight favoring of the pathway to Cpds. F and E being seen. Significantly more 17-hydroxyprogesterone was converted to Cpds. F and E than to Subst. S (P.003; Table 2).

With DOC as substrate, Cpd. B and Unknown Steroids No. II and No. and No. III were the major conversion products. Cpd. B was formed at the rate of 19.11% of the original substrate in 2 hours of incubation. This amount represented 58.73% of the total recovered steroid and indicated a significant favoring of the enzymatic conversion of DOC to Cpd. B ($P < .001$; Table 2).

Cell-Free Adrenal Homogenates. Our initial chromatograms of homogenate incubations were obscured by the presence of a large ultraviolet absorbing area which would not move from the origin of the chromatogram, and which could not be removed by solvent partition (70% ethanol-petroleum ether), by adsorption on charcoal or by overrunning the papergram in a hexane tank with this solvent. Heard *et al.* (17) also observed difficulties with this cytoplasmic phospholipid-like material which caused a "smearing" of their initial papergrams. A partitioning method was adopted (petroleum ether-water with subsequent extraction of the aqueous phase with methylene chloride) which removed this cytoplasmic interfering material and allowed for "clean" chromatograms in a single run.

The substrates which produced no steroids when incubated with adrenal slices were tried in cell-free adrenal homogenate preparations. The results of 21 separate incubations are summarized in Table 3. Substrates which did not yield detectable amounts of conversion products with adrenal slices did produce small amounts of alpha-ketolic steroids when incubated for 3 hours with the fortified, sucrose supernatant of a cell-free adrenal homogenate. An average of 2.6 ± 1.4 μg. Cpd. E and 0.67 ± 0.76 μg. Cpd. A were produced from endogenous substrates by incubation of this adrenal homogenate prepared from 57–83 mg. of tissue. This endogenous production of Cpd. E and Cpd. A is subtracted from that produced from the other substrates to get the net steroid produced from each substrate. In all cases except the squalene incubation, these substrates produced more Cpd. E than Cpd. A while no Cpd. F or Cpd. B was produced endogenously or from any of these substrates except cholesterol.

The amount of Cpd. E and Cpd. A recovered with acetate (in concentrations of 1, 5, 10, 100 or 1000 μmoles) as the substrate was in the same range

TABLE 4. AMOUNTS AND RADIOACTIVITY OF ALPHA-KETOLIC STEROIDS PRODUCED BY INCUBATION OF ACETATE-1-C^{14} AND CHOLESTEROL-4-C^{14} WITH A CELL-FREE ADRENAL HOMOGENATE PREPARATION

Flask #	Adrenal wt.[a] mg.	Incubate preparation[b,c]		Alpha-Ketolic steroids recovered[f]—specific activity											
		Substrate[d]	C^{14} μcuries	μg. F	Net C.P.M.[e]	Sp. act. C.P.M./μg.	μg. E	Net C.P.M.	Sp. act. C.P.M./μg.	μg. B	Net C.P.M.	Sp. act. C.P.M./μg.	μg. A	Net C.P.M.	Sp. act. C.P.M./μg.
#160	114	460 μg. Cholesterol-C^{14}	1.15	13.65	3111.90	227.98	16.40	4546.83	277.25	12.47	4060.85	325.58	23.23	9906.24	427.44
#161	114	10 μmoles Acetate-C^{14}	2.20	2.5	—	—	10.00	—	—	—	—	—	4.5	—	—
#162	114	400 μg Cholesterol 10 μmoles Acetate-C^{14}	2.20	6.0	—	—	11.0	—	—	4.5	—	—	9.4	—	—
#163	114	400 μg. Pregnenolone 460 μg. Cholesterol-C^{14}	1.15	19.95	102.34	5.13	19.95	243.65	12.21	25.59	531.92	20.79	38.71	4245.90[g]	109.68

[a] Adrenals pooled from 16 male hamsters, wet weight before homogenation recorded.
[b] Cell-free homogenate in .25 M sucrose—see Table 3.
[c] Time of incubation = 3 hrs.
[d] Steroid substrates added 4 μg./mg. adrenal.
[e] Net C.P.M. = counts per minute of steroid spot less C.P.M. of adjacent segment of chromatogram.
[f] μg. of steroid recorded for #160 and #163 are based on 76.2% recovery after acetylation.
[g] Estimated from the Cpd. A area on the chromatogram although this spot did not separate well from the material running with the solvent front.

(3.4 ± 1.2 μg. Cpd. E and a trace of 1 μg. Cpd. A). Significantly, this production of steroid with acetate added does not differ from that produced by incubation from endogenous sources; thus, there appears to be no net production of alpha-ketolic steroids from acetate by this homogenate preparation under the conditions of this experiment. There is a net production of Cpd. E and Cpd. A from squalene, cholesterol or cholestan-3-one when these substrates are incubated with cell-free adrenal homogenate preparations. With cholesterol as substrate, there was 6.44 ± 1.9 μg. Cpd. E and 3.31 ± 1.2 μg. Cpd. A produced. This is a net production of 3.84 μg. ($P = .011$) and 2.64 μg. ($P = .006$) of Cpds. E and A respectively (Table 3).

C^{14}-Labeled Cholesterol and Acetate in Homogenates. Table 4 shows the amounts and radioactivity of alpha-ketolic steroids recovered after incubation of C^{14}-labeled acetate and cholesterol with the cell-free homogenate preparation. From Flask #161, in which 10 μmoles of acetate-1-C^{14} were incubated as substrate, 2.5 μg. Cpd. F, 10.0 μg. Cpd. E and 4.5 μg. Cpd. A were recovered. None of these corticoids were radioactive, indicating that there was no utilization of the labeled acetate. When 10 μmoles of labeled acetate plus 400 μg. of non-radioactive cholesterol were added as substrates, greater amounts of steroid (6.0 μg. Cpd. F, 11.0 μg. Cpd. E, 4.5 μg. Cpd. B and 9.4 μg. Cpd. A) were recovered (Flask #162); none of the recovered corticoids showed any radio-activity (Table 4). The data would indicate that the amounts of steroid recovered from Flask #161 were produced from endogenous substrates in the adrenal homogenate preparation and that the greater amounts of steroid recovered from Flask #162 were derived from the non-radioactive cholesterol also added as substrate.

When cholesterol-4-C^{14} was used as the substrate (Flask #160), large amounts of Cpds. F, E, B and A were recovered. Each of these four corticoids showed considerable radioactivity and all of about the same specific activity, (Table 4). When labeled cholesterol plus non-radioactive pregnenolone (Flask #163) were added as substrates there were greater yields of each of the corticoids but considerably less radioactivity in each (Table 4). The greater yield of corticoids from incubation Flask #163 is explained by the fact that pregnenolone is readily converted to these steroids by the homogenate preparation used. The considerably lower specific activity of the steroids produced from Flask #163 when compared to that produced from Flask #160 (where labeled cholesterol alone was the substrate) may be explained by the direct conversion of pregnenolone to the steroids as well as the dilution of the cholesterol radioactivity by conversion of the cholesterol to pregnenolone in the pathway of corticosteroidogenesis. After determination of radioactivity in each of the steroids from Flasks #160 and #163, the corticoids were eluted with methanol, acetylated separately and rechromatographed in the NB_1 system. Radioactivity was again counted for each steroid and is summarized in Table 5. Acetylation of Cpd. B from each flask led to the formation of Cpd. B acetate of approximately the same

TABLE 5. AMOUNTS AND RADIOACTIVITY OF STEROIDS AND THEIR ACETATE DERIVATIVES PRODUCED BY INCUBATION OF C^{14}-CHOLESTEROL (OR C^{14}-CHOLESTEROL PLUS NON-RADIOACTIVE PREGNENOLONE) WITH A CELL-FREE HAMSTER ADRENAL HOMOGENATE PREPARATION

Free steroids				After acetylation				
				Steroid acetate		Unknown acetates		
Steroid	Flask no.	Amount μg.	Sp. Act. C.P.M./μg.	Amount μg.	Sp. Act. C.P.M./μg.	Designation	Amount/ μg.	Sp. act. C.P.M./μg.
Cpd. F	#160[a]	13.65	227.98	5.0	308.55	"Y"	5.4	Not measured
	#163[b]	19.95	5.13	7.7	9.52	"Y"	7.5	Not measured
Cpd. E	#160	16.40	277.25	9.5	259.73	"Z"	3.0	160.26
	#163	19.95	12.21	1.0	9.88	"Z"	15.2	21.31
Cpd. A	#160	23.23	426.44	12.7	479.71	"X"	5.0	769.71
	#163	38.71	109.68	17.5[c]	93.33	"X"[c]	12.0	200.2
Cpd. B	#160	12.47	325.58	9.5	337.58			
	#163	25.59	20.79	19.5	23.54			

[a] = Cholesterol-4-C^{14} as substrate.
[b] = Cholesterol-4-C^{14} plus non-radioactive pregnenolone as substrate.
[c] = Rechromatographed in another system (NB_1) but not acetylated.

specific activity as the free steroid. Acetates of unknown Alpha-ketols ("Y", Rf. 0.56; "Z", Rf. 0.85; "X", Rf. 0.81) separated from the acetates of Cpds. F, E and A respectively (Table 5). These unknowns were not identified further.

C^{14}-Labeled Acetate and Cholesterol in Vivo. Available windowless gas flow strip counters were not sensitive enough to detect the radioactivity on the chromatograms of our adrenal effluent blood samples; therefore the Cpd. F spots were located by ultraviolet reflex photography and the entire strip cut into 3–3.5 cm. segments. Care was taken to include all of the Cpd. F on one segment. Each segment was eluted with methanol and plated out on glass coverslips. All of the radioactivity was located in Cpd. F except for the tail end of the chromatograms where non-corticoid, ultraviolet absorbing material runs with the solvent front.

TABLE 6. RADIOACTIVITY OF CPD. F ISOLATED FROM ADRENAL VENOUS BLOOD OF HAMSTERS AFTER I.V. INJECTION OF ACETATE-1-C^{14} OR CHOLESTEROL-4-C^{14}

Treatment	Ave. body wt. gm.	No. in group	Ave. adrenal wt. mg.	Total time collected, hr.	Total blood vol. ml.	μg. F	Net C.P.M.[c,d]	Sp. act. C.P.M./μg.	Flow rate ml./hr./100 g. B.W.	Secretion rate μg./100 mg.[g]/hr.	Secretion rate μg./adrenal/100 g. B.W./hr.	Concentration μg. F/ml. blood
C^{14}-acetate[a]	146.8	4	13.6	3.33	6.5	12.5	94.44[e]	7.55	1.33	27.64	2.55	1.92
C^{14}-cholesterol[b]	159	5	16.9	6.10	12.9	20	—[f]	—	1.33	19.45	2.06	1.55

[a] 10 μcuries sodium acetate-1-C^{14} (sp. act. 1 millicurie/millimole) in saline injected via the femoral vein into each animal after adrenal vein cannulated.
[b] 10 μcuries cholesterol-4-C^{14} (sp. act. 19.35 millicuries/millimole) in 10% alcoholic saline suspension injected via the femoral vein into each animal after adrenal vein cannulated.
[c] Net C.P.M. = counts per minute of steroid less C.P.M. of adjacent segment of chromatogram.
[d] All counts made for at least one hour in windowless gas flow counter. Steroid spots and paper segments were eluted with methanol and plated out on glass coverslips which were then placed in planchets for counting.
[e] This value is >4 times background.
[f] Insignificant counts.
[g] 100 mg. = 100 mg. adrenal wt.

Table 6 shows the results of the pooled adrenal venous blood samples obtained from male hamsters which were injected at the time of cannulation via the femoral vein with acetate-1-C^{14} or cholesterol-4-C^{14}. Only Cpd. F was recovered from the adrenal venous blood. From C^{14}-acetate injected hamsters 12.5 μg. of Cpd. F was recovered, showing 94.44 net C.P.M. with a specific activity of 7.55 C.P.M./μg. Cpd. F. In a similar experiment, there was no significant radioactivity in 20 μg. of Cpd. F isolated from the adrenal venous blood of hamsters injected with Cholesterol-C^{14}.

DISCUSSION

The results of this study indicate that hamster adrenal glands contain little or no stored cortical hormones. There is no detectable production of cortical steroids from endogenous substrates from whole cell preparations, i.e., when adrenal slices are incubated *in vitro* under the conditions of these experiments. In cell-free suspensions, the equivalent of 80–100 mg. of adrenal tissue produces only minute amounts of steroids (2–3 μg. of Cpd. E plus Cpd. A) in 3 hours of incubation even when supplied with additives and enzymatic cofactors. It is suggested that hamster adrenals not only contain no stored hormone, but that there is also a paucity of stored precursor material. These findings are in contrast to that of other species studied.

The fact that there were no hormones stored in the gland nor any *in vitro* hormone production from endogenous substrates made this preparation suitable for studying the enzymatic ability of adrenal slices to carry out certain reactions on chosen steroid substrates which could be added to the incubation flask. Any corticoids isolated after incubation of adrenal slices with a suspected steroid precursor must have been produced by enzymatic conversion of this precursor. When pregnenolone was used as the steroid substrate, Cpds. F, E and B were isolated after the incubation period. These incubation products, as well as those produced from other steroid substrates, indicate the presence of the 3β-ol dehydrogenase, the 17-hydroxylase, the 21-hydroxylase and the 11β hydroxylase enzyme systems in hamster adrenal slices. With progesterone as the substrate, there is approximately an equal formation of Cpd. F and Cpd. B, which agrees with the thesis that progesterone is the "key" compound and may be hydroxylated at C-17, in which case it would go on to form Cpd. F via Subst. S, or it may be hydroxylated at C-21, in which case it would go on to form Cpd. B via DOC (8). It is interesting to note that no DOC or Subst. S was recovered from progesterone incubations. This would indicate that the reactions to the final products (Cpd. B and Cpd. F respectively) went essentially to completion. Cpd. F and also Subst. S were recovered after incubation with 17-hydroxyprogesterone. The recovery of the intermediate product (Subst. S) was unexpected and may indicate that the 11-β-hydroxylation was the limiting step in this sequence.

Incubations of adrenal slices with DOC as substrate produced predominantly Cpd. B. In addition, there were two other alpha-ketolic steroids produced which had the chromatographic mobility of Cpd. F and Cpd. E respectively in Bush's toluene-methanol-water system. These substances we have called Unknown Steroids No. II and No. III respectively because further characterization indicated that they were not Cpd. F or Cpd. E. These unknowns are seen as trace amounts after incubations with other substrates (e.g., progesterone; Table 1); however, only after DOC incubations are they produced in relatively large amounts (33% of the total converted steroid recovered and 10.5% of the original substrate). Conversion of DOC to Cpd. B by hog, ox and rabbit adrenal tissue does not apparently involve the additional formation of such relatively large amounts of unknown alpha-ketolic steroids (17). The biosynthesis of aldosterone has not been definitely established; however, Wettstein *et al.* (18) have suggested that DOC may be its immediate precursor. Sufficient amounts of authentic aldosterone were available to determine its chromatographic mobility in the toluene-methanol-water and E_2B systems. Neither Unknown No. II nor No. III was chromatographically identical to aldosterone. Further identification of these interesting Unknowns was not attempted at this time.

Incubation of cholesterol, cholestan-3-one or squalene with adrenal slices (whole cell preparations) produced no alpha-ketolic steroids, and even when the incubation was fortified with the "standard cofactor additions" there were only slight unmeasurable amounts of Cpd. E and Cpd. A produced. In the whole cell preparations, delta-4-cholesten-3-one was converted to the corticoid end products, indicating that the side chain could be degraded to 21 carbons. With cell-free adrenal homogenate preparations, these same substrates (cholesterol, cholestan-3-one and squalene) were readily converted to corticoid end products. This was confirmed by the formation of radioactive Cpds. F, E, B and A from cholesterol-4-C^{14}. These findings indicate that the production of adrenal corticoids from cholesterol by cell-free homogenate preparations (reactions that would not proceed in the original whole cell adrenal slice preparations without added cofactors) may depend upon permeability of the cholesterol, and thus the availability of the substrate to be acted upon.

Although there were small amounts of Cpd. E and Cpd. A formed when varying concentrations of sodium acetate were added as substrate to the homogenate preparation incubation (Table 3), these amounts were not significantly higher than those isolated from the preparation after incubation when no substrate was added, i.e., the amount of Cpd. E and Cpd. A produced from endogenous sources. This finding that acetate was not converted to adrenal corticoids by the homogenate preparation was confirmed by using acetate-1-C^{14}. In these experiments (Table 4) although Cpd. F, Cpd. E and Cpd. A were isolated after incubation, there was no radioac-

tivity in any of these steroids. Similar results, i.e., the failure of labeled acetate to be converted to labeled adrenal corticoids, were observed by Hofmann and Davison (19) and independently in our own laboratory (20), both using rat adrenal slices, and also by Reich and Lehninger (21) using beef adrenal homogenates. The results of the latter homogenate preparation were similar to our hamster adrenal homogenate results also in the fact that labeled cholesterol was converted to labeled corticoids. In contrast to this, other investigators have demonstrated the conversion of acetate-C^{14} to radioactive adrenal steroids using hog slices (22, 23, 24), cow and bull slices (22) and washed beef homogenate preparations (9). The differences obtained by various workers in the success or failure of their systems to incorporate C^{14}-acetate into adrenal corticoids *in vitro* may be due to technical problems involved in the various systems as well as actual species differences in the ability of the adrenals to perform this conversion *in vitro*.

In other species, the endogenous substrates used for steroid production are generally considered to be cholesterol, especially during stress or otherwise when the adrenals are stimulated by ACTH, and perhaps acetate, the conversion of which may not be stimulated by ACTH (25). Stimulation of the adrenals of most species to produce adrenal corticoids, as by stress, epinephrine or ACTH, results in a sharp fall in adrenal cholesterol (26, 27, 28). This decrease of adrenal cholesterol in stressed rats, for example, is a decrease only of the esterified portion of the cholesterol while the free adrenal cholesterol remains fairly constant (27, 28). Total adrenal cholesterol in the rat ranges from 3.57 to 4.96 mg./100 mg. adrenal (28); 90% of this total cholesterol is in the esterified form (27, 28). Hamster adrenal cholesterol levels have been reported as 0.152 to 0.175 mg./100 mg. adrenals (mg.%) in males and 0.311 to 0.338 mg.% in females (28), and 0.29 ± 0.03 mg.% (29). Zero to 8% of this cholesterol is in the esterified form in the hamster adrenal. Thus, free cholesterol levels are about the same in the hamster and the rat, but the rat adrenal contains about 9 times more esterified cholesterol than the hamster adrenal (28). This latter finding is important inasmuch as hamster adrenals are relatively lacking in esterified cholesterol and it is this fraction that fluctuates in response to stress in rats. Hamsters exposed to cold stress (5° C) for varying periods of time showed no change in adrenal cholesterol levels (28). Amphenone B, a compound which inhibits adrenal corticoid secretion in the dog (30), calf (31), and man (32, 33, 34), causes a large accumulation of cholesterol in the rat adrenal which does not decrease when the animal is subjected to cold stress (35). This evidence is interpreted as indicating that cholesterol, the endogenous precursor of corticoids, accumulates in the adrenal because of a block (or series of blocks) farther on in the steroid biosynthesis sequence. In the amphenone-treated hamster, on the other hand, cholesterol does not accumulate in the adrenals and corticoid production (measured indirectly

by urinary Porter-Silber chromogens) seems to be normal (29). These findings concerning the lack of esterified cholesterol in the hamster adrenal, and the lack of cholesterol accumulation or corticoid production impairment after amphenone treatment, suggest that this species may not utilize the usual method of steroid synthesis. In several experiments of this study, digitonin, a cholesterol precipitant (0.025 ml. of a 1% solution) added to the adrenal homogenate preparation failed to inhibit the formation of corticoids from endogenous substrates. Further suggestive evidence that cholesterol is not a corticoid precursor in the hamster is presented by the demonstration reported here that intravenously injected cholesterol-4-C^{14} does not produce any radioactive labeling of the Cpd. F isolated by direct cannulation of the adrenal vein and analysis of the effluent blood collected. It must be pointed out, however, that the endogenous cholesterol pool may be so large as to be diluting out the infused cholesterol-4-C^{14} with the result that any cortisol-4-C^{14} formed might not have been detected.

The nature of cholesterol as a corticoid precursor in other species is too well established to be questioned as a fundamental hypothesis. These apparent contradictions observed in our studies on hamster adrenals do not question the basic foundation of cholesterol as a precursor, but merely indicate that the use of cholesterol as a predominant corticoid precursor is not a universal phenomenon and the hamster with its histochemically "lipid poor" adrenal may be an exception. In *in vitro* slice incubates or in *in vivo* infusion experiments, cholesterol is faced by the whole, intact hamster adrenocortical cell and apparently can not be utilized for synthesis of steroids. When the cell membrane is disrupted in the formation of homogenates, cholesterol is readily utilized. The basic unique difference in steroidogenesis by hamster adrenocortical cells appears to be a relative impermeability of the cell membrane to cholesterol, resulting in the synthesis of cortical steroids from other precursors, the nature of which remains to be identified more fully. The rapid *in vivo* incorporation of infused C^{14}-acetate and delivery of radio-labeled Cpd. F. strongly suggests acetate as a predominate corticoid precursor in the hamster. The results of these experiments do not eliminate the possibility that acetate enters the adrenal cortical cell and is incorporated into cholesterol, and that intracellular cholesterol thus formed may be a corticoid precursor.

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PLASMA LEVELS OF DEHYDROEPIANDROSTERONE IN THE DOG[1]

GEORG W. OERTEL AND KRISTEN B. EIK-NES

Department of Biochemistry, University of Utah College of Medicine, Salt Lake City, Utah

ABSTRACT

Low circulating levels of plasma dehydroepiandrosterone have been found in the dog. A negligible adrenocortical secretion of this steroid apparently takes place in this animal species.

Following the intravenous administration of pregnenolone and 17α-hydroxypregnenolone to the dog, high plasma levels of conjugated dehydroepiandrosterone have been found. The tissues in the dog leg *may* perform such a conversion. Different theories pertinent to the origin of plasma dehydroepiandrosterone in the dog have been discussed, since dogs lacking adrenals, testis and liver can still convert tritiated pregnenolone to tritiated dehydroepiandrosterone.

WHEREAS several C-21 steroids have been found in adrenal vein or in systemic blood of the dog (1, 2, 3) relatively little is known about blood plasma concentrations of C-19 steroids in this animal species (4, 5).

Paschkis *et al.* (6) observed that dogs, regardless of sex, excreted from 0.5–1.5 mg. 17-ketosteroids in urine per 24 hours and when dogs were given testosterone propionate, no increase in the secretion of urinary 17-ketosteroids occurred. Glenn and Heftmann (7) concluded that the normal dog excreted less than 1 mg. ketosteroid in the urine per day and could not obtain an alteration of 17-ketosteroid excretion when normal dogs were given adrenocorticotropic hormone (ACTH).

In the normal man, from 10–15 mg. of 17-ketosteroids are excreted per 24 hours and an increased urinary concentration of these steroids can be found following ACTH and testosterone administration (8, 9). Thus a difference may exist between man and dog in either production or catabolism of 17-ketosteroids. In the present communication we wish to report the formation of dehydroepiandrosterone by the hind leg of the dog. Further, we have tested the theory of Lieberman and Teich (10) that 17α-hydroxy pregnenolone may be a precursor for dehydroepiandrosterone.

METHODS

A. *Animal preparations*

1) *Cannulation of the liver:* This was done as previously described from our laboratory (11).

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One hour after the operation, experiments were started. Fifty mg. pregnenolone dissolved in 10 ml. propylene glycol[2] were injected over 2–3 minutes by the cannula in the portal vein. Blood for steroid determination was obtained through the cannula in the hepatic vein with 10 minute intervals over one hour, and systemic blood was withdrawn from the femoral vein over the same period using the same timing as for the hepatic vein samples.

2) *Adrenal cannulation:* The method of Zaffaroni and Burton was used (12). Adrenal vein blood was collected in 10 minute samples before and after the intravenous injection (front leg vein) of 50 mg. pregnenolone over 2–3 minutes. A systemic blood specimen was obtained from the right femoral vein every time an adrenal vein sample was collected.

3) *Preparation of the isolated dog leg:* The left femoral artery and vein were reached in the *angulus femoralis* before they entered the abdominal cavity. A small tributary of the femoral artery was dissected and a No. 24 needle attached to polyethylene tubing inserted into this arterial branch in such a fashion that the end of the needle tipped into the lumen of the femoral artery without interfering with the arterial irrigation of the leg. The femoral vein was then dissected free and followed into the abdominal cavity through a blunt abdominal incision. A detailed dissection was done of the venous plexus, and a loose ligature was placed around the vein above the dissected plexus. Care was taken to prevent stasis of the leg. A polyethylene tube was inserted into the femoral vein through one of the branches of the plexus. All branches of the plexus not coming from the leg were severed between double ligatures and the major lymphatic drainage from the leg was finally blocked. During experiments the loose ligature around the femoral vein was tied off and pregnenolone or 17α-hydroxy pregnenolone[3] injected by the cannula leading into the femoral artery. The cannula in the femoral vein was opened and the venous blood leaving the leg collected. During experiments lasting for more than 10 minutes, citrated dog blood was given intravenously by the left jugular vein at a rate of about 35–45 ml. per minute. Several experiments were conducted to test if the described preparation allowed administered steroids to escape into the systemic circulation.

4) *Preparation for hepatectomy, adrenalectomy and removal of the testis in the dog:* Through a midline incision as described for cannulation of the liver (11) the liver and the intestines were reached. Evisceration was done and a siliconed pyrex glass cannula introduced into the inferior vena cava (13). This allowed the complete removal of the liver. The adrenals and both testes were then removed and the experiments started. This preparation was extremely difficult to handle. The animal needed blood transfusion immediately after the abdominal incision, and an increased survival rate was seen if 25 mg. of cortisol were added to the infusion blood after adrenalectomy. After considerable experience with this preparation, the mortality is still 48%.

B. *Determination of steroids*

a) *In plasma:* The estimation of plasma dehydroepiandrosterone was carried out by the method of Oertel and Eik-Nes (14). Hydrolysis of plasma DHEA conjugates by commercial preparations of phosphatases has been described (15). The steroids liberated by this procedure were extracted with 3×1 volume ethyl acetate (EtOAc). After evaporation of remaining traces of organic solvent *in vacuo*, the aqueous phase was brought up to the original volume of plasma by adding H_2O, adjusted to pH 1.8 by means of 1 *N* HCl and incubated with 1 mg. pepsin/ml. solution for 15 hours at 37°C. The sample was then processed in accordance with the method of Oertel and Eik-Nes (14).

[2] This preparation was supplied by the Ciba Pharmaceutical Products, Inc. Summit, New Jersey.

[3] Obtained from Glidden Company, Chicago 39, Illinois.

b) *In tissues:* Nervous and connective tissue were cut into small pieces, ground, and continuously extracted with ethanol (EtOH) for 4 hours. The other tissues were homogenized in a Waring Blender with 10 volumes EtOH/gm. of tissue. An equal volume of EtOH was added and the suspension was refluxed for 2 hours, centifuged and the supernatant decanted. After washing 2× with EtOH the residue was discarded and the total extract evaporated to dryness *in vacuo*. By distribution of the residue between 80% methanol and hexane, most of the lipid material was removed. The aqueous MeOH was evaporated *in vacuo*, the residues suspended in water and hydrolyzed with H_2SO_4. The hydrolysate was extracted and processed by the method of Oertel and Eik-Nes (14).

C. *Identification of dehydroepiandrosterone following the administration of pregnenolone by the isolated dog leg*

a) *Tritiated pregnenolone:*[4] After administration of randomly labeled tritiated pregnenolone, the plasma samples were processed in accordance with the method of Oertel and Eik-Nes (14). After chromatography on paper, the spots exhibiting the running rate of dehydroepiandrosterone were eluted and evaporated. A 5% aliquot was dissolved in chloroform-beeswax and counted in a windowless counter with approximately 10% efficiency. The rest of the material was acetylated with acetic anhydride-pyridine and chromatographed with standard dehydroepiandrosterone acetate in propylene-glycol/methylcyclohexane (16). The area on the sample strip corresponding to that of dehydroepiandrosterone acetate was eluted, a 5% aliquot counted and the rest of the sample chromatographed in formamide-hexane/benzene. Material with an Rf of dehydroepiandrosterone acetate was eluted, and a 5% aliquot counted. To the rest of the material authentic dehydroepiandrosterone acetate was added. This mixture was recrystallized three times from hexane. Each time, the crystalline material obtained was dissolved in 10 ml. of acetone and 0.1 volume was submitted to a color reaction characteristic for $\Delta 5$-3β-hydroxysteroids (17) while 0.1 volume was counted in a windowless counter.

The blood plasma obtained from gonadectomized adrenalectomized and eviscerated dogs given randomly labeled tritiated pregnenolone was processed by the standard method for the determination of plasma dehydroepiandrosterone (14). After paper chromatography and elution, synthetic dehydroepiandrosterone was added and the sample was partitioned in a system consisting of equal volumes of equilibrated 47.5% ethanol in water and 40% ethyl acetate in hexane. The Zimmermann reaction was performed on each solvent phase and the radioactivity in each phase was also determined. The partition coefficient for dehydroepiandrosterone and pregnenolone in this system is 3.1 and 3.8 respectively.

b) *Non-labeled pregnenolone:* After extraction, hydrolysis and purification of the plasma (14) the final extract was submitted to Girard Separation (18, 19). The ketonic material was chromatographed twice in formamide-hexane/benzene and material with an Rf value of dehydroepiandrosterone eluted. This material was subjected to chromatography in propylene glycol/methylcyclohexane and material behaving like authentic dehydroepiandrosterone was eluted and divided into three aliquots.

The first aliquot was dissolved in concentrated H_2SO_4. Its absorption spectrum was recorded and compared to the sulfuric acid spectrum of authentic DHEA (20).

The second aliquot was acetylated with C^{14}-acetic anhydride-pyridine. Fifty μg. standard DHEA were acetylated in the same fashion. Both acetylated samples were chromatographed in formamide-hexane/benzene and subsequently in propylene glycol

[4] Obtained through the courtesy of Dr. Marcel Gut, Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts.

TABLE 1. CONCENTRATION OF DEHYDROEPIANDROSTERONE AND 17-HYDROXYCORTICOSTEROIDS* (17-OHCS) IN ADRENAL VEIN BLOOD OF FOUR DOGS 30–60 MINUTES AFTER THE "STRESS" OF LAPAROTOMY (23)

Sex	Plasma 17-OHCS μg./10 kg. body weight/min.	Plasma dehydroepiandrosterone μg./10 kg. body weight/min.
M	6.3	.5
M	12.2	not detectable
F	7.4	.5
F	8.4	.5

* Determined by the method of Eik-Nes (31).

/methylcyclohexane. The location of the C^{14} labeled compounds was determined by a strip counter.

The third aliquot was divided in two parts. One part was submitted to the Zimmermann reaction as modified by Wilson and Fairbanks (21) and the other part to the reaction of Allen *et al.* (22). In both cases the absorption spectra of the color complexes were compared to those obtained with standard DHEA.

All steroid levels except those in Table 2 are from dogs anesthetized with sodium pentobarbital.

RESULTS

In Table 1 are recorded adrenal vein concentrations of 17-hydroxycorticosteroids and dehydroepiandrosterone in "stressed" dogs (23). A high secretion rate of 17-hydroxycorticosteroids was found, whereas the adrenal output of dehydroepiandrosterone was too low to be measured adequately, since the Zimmermann curves from 40–60 ml. of adrenal vein blood extract were far from conclusive (14). Extremely low levels of dehydroepiandrosterone were found in blood plasma from the systemic circulation in dogs (Table 2). However, these levels increased following the administration of pregnenolone (Table 2).

In order to determine whether the conversion of pregnenolone to dehydroepiandrosterone was done by the canine adrenal cortex, 50 mg. of

TABLE 2. PLASMA LEVELS OF DEHYDROEPIANDROSTERONE (μg./100 ML.) IN NORMAL DOGS AFTER THE INTRAVENOUS INJECTION OF 10 ML. STERILE PROPYLENE GLYCOL OR 10 ML. STERILE PROPYLENE GLYCOL CONTAINING DIFFERENT AMOUNTS OF PREGNENOLONE

Dog	Treatment	Control	Plasma dehydroepiandrosterone Hours after injection		
			1	2	4
A	Propylene glycol	0.9	*	0.90	0.8
B	Propylene glycol	0.5	0.4	0.6	0.7
C	50 mg. pregnenolone	*	97.0	68.0	*
D	50 mg. pregnenolone	*	44.0	27.0	*
E	50 mg. pregnenolone	1.3	*	44.0	36.0
F	5 mg. pregnenolone	3.0	19.0	16.0	13.0
G	5 mg. pregnenolone	1.9	*	14.0	10.0
H	5 mg. pregnenolone	2.0	10.0	10.0	9.0

* Not measured.

TABLE 3. PLASMA LEVELS OF DEHYDROEPIANDROSTERONE AFTER THE INTRAVENOUS INJECTION OF PREGNENOLONE OR 17α-HYDROXYPREGNENOLONE INTO THE ISOLATED DOG LEG (ALL STEROID LEVELS EXPRESSED IN μg./TOTAL PLASMA SAMPLE OBTAINED)

Dog	Steroid injected	Time after injection (minutes)	Volume of blood plasma obtained during this period (ml.)	Dehydroepiandrosterone μg/total plasma sample
P (29 kg.)	Pregnenolone 50 mg.	0– 5	92	29
		5–12	89	32
		12–21	101	30
		21–30	93	23
		30–42	84	16
		42–50	78	29
		50–60	64	56
Q (31 kg.)	17α-hydroxypregnenolone 10 mg.	0– 2	129	16
		2– 6	114	35
		6–11	98	26
		11–15	96	23
		15–22	105	25
		22–32	104	27
		32–41	114	30
		41–51	94	27
		51–61	49	10

Plasma concentrations of dehydroepiandrosterone in venous blood of normal dogs not given pregnenolone or 17α-hydroxypregnenolone have been found to range from 1–3 μg. per 100 ml. (Cf. Table 2.)

pregnenolone were injected intravenously into a dog in which the right adrenal gland had been removed and the left adrenal gland cannulated. The levels of dehydroepiandrosterone in most samples of adrenal venous effluent did not exceed those of systemic blood plasma collected simultaneously.

When 50 mg. of pregnenolone were injected by the portal vein of the dog, the concentration of dehydroepiandrosterone in hepatic venous blood was at no time considerably higher than that of the femoral vein.

Since the experiments failed to show that either the adrenal cortex or hepatic tissue were the predominant sites of conversion of pregnenolone to dehydroepiandrosterone, the ability of the hind leg in the dog to convert pregnenolone to plasma dehydroepiandrosterone was tested. Table 3 clearly demonstrates that the isolated dog leg is capable of transforming

TABLE 4. CARBON^{14} IN ETHANOLIC EXTRACTS OF PLASMA FROM DOGS GIVEN DIFFERENT C^{14} LABELED STEROID HORMONES BY THE ISOLATED DOG LEG. ALL BLOOD SAMPLES WERE OBTAINED SIMULTANEOUSLY

Hormone administered by the femoral artery	*Counts/minute/10 ml. blood plasma extract		
	Time after administration (min.)	Vein blood from the isolated leg	Vein blood from the jugular vein
1,000,000 cpm testosterone-4-C^{14}	10	3635	not detectable
1,000,000 cpm cortisol-4-C^{14}	30	1626	8
1,000,000 cpm cortisol-4-C^{14}	60	1026	6
1,000,000 cpm progesterone-4-C^{14}	20	1610	2

* Extracts counted in a windowless counter.

pregnenolone and 17α-hydroxypregnenolone to plasma dehydroepiandrosterone.

Of course, the possibility exists that the isolated dog leg preparation leaks and hence allows the re-entrance of steroids by the intact arterial supply. To test this, several experiments were done utilizing different radioactive steroids. Tables 4 and 5 indicate that this leakage is minimal.

Using the isolated dog leg preparation, 10 mg. tritium labeled pregnenolone with 10^6 disintegrations/minute/mg. were given. The plasma dehydroepiandrosterone isolated from the venous effluent over 53 minutes gave 7570 cpm. At an efficiency of 10% this corresponds to 75,700 disintegrations/minute. Hence, the total conversion of pregnenolone to plasma dehydroepiandrosterone by the isolated dog leg over the period tested amounted to 0.79%. In another experiment on the isolated dog leg a con-

TABLE 5. RADIOACTIVITY (CPM/10 G. TISSUE) IN ETHANOL EXTRACT OF TISSUE HOMOGENATES FROM DIFFERENT TISSUES OBTAINED FROM THE ISOLATED DOG LEG AND THE CONTROL LEG 60 MINUTES AFTER THE INTRA-ARTERIAL ADMINISTRATION OF 1,000,000 CPM TRITIUM LABELED PREGNENOLONE BY THE ISOLATED LEG

Tissue	Counts/minute/10 gm. tissue	
	Isolated leg	Control leg
Femoral artery	135	not detectable
Fat	100	2
Femoral nerve	197	not detectable
Lymphatic tissue	210	5
Femoral vein	130	2
Muscular	198	3

version of labeled pregnenolone to plasma dehydroepiandrosterone of 1.09% was found.

The identity of dehydroepiandrosterone isolated in these two experiments was proven by running rates of the free and acetylated compounds in different systems of paper chromatography, and by crystallization of the acetate to constant specific activity after dilution with non-labeled dehydroepiandrosterone acetate. After the first recrystallization the specific activity was found to be 1.13 cpm/μg. dehydroepiandrosterone acetate, after the second crystallization 1.03 cpm/μg. and after the third crystallization a specific activity of 1.24 cpm/μg. DHEA acetate was obtained.

By similar paper chromatographic criteria, and also by partition behavior in H_2O/ethanol-ethyl acetate/hexane, dehydroepiandrosterone was identified in plasma of two eviscerated, adrenalectomized and gonadectomized dogs obtained 35 minutes after the administration of pregnenolone I.V.

The identity of dehydroepiandrosterone in the venous effluent of the isolated dog leg after administration of non-labeled pregnenolone was

TABLE 6. COMPARISON BETWEEN THE COMPOUND OBTAINED FROM ISOLATED DOG LEG PLASMA AFTER ADMINISTRATION OF 50 MG. PREGNENOLONE AND STANDARD DEHYDROEPIANDROSTERONE. A TOTAL AMOUNT OF 600 ML. PLASMA WAS PROCESSED

Criteria	Results	
	Plasma compound	*Authentic DHEA*
1. Paper chromatography:		
Formamide/hexane-benzene (1:1 v/v)	R_F 0.72	R_F 0.73
Propylene glycol/methyl-cyclohexane	R_T* 1.86	R_T 1.88
1a. After acetylation.		
Formamide/hexane-benzene (1:1 v/v)	R_F 0.92	R_F 0.92
Propylene glycol/methyl-cyclohexane	R_T 4.16	R_T 4.09
2. Zimmermann reaction:		
Absorption maximum	508 mμ	510 mμ
3. Allen's reaction:		
Absorption maximum	602 mμ	602 mμ

* R_T = relative mobility referring to standard testosterone.

demonstrated by running rates of the free compound and its C^{14} labeled acetate in different systems of paper chromatography (Table 6), by its absorption curves in the Zimmermann and Allen reaction and by its sulfuric acid chromogen spectrum (Fig. 1).

Apparently, dehydroepiandrosterone leaves the isolated leg in conjugated form (Fig. 2) and without hydrolysis *no dehydroepiandrosterone could be detected by our method in adrenal, hepatic, or peripheral blood of any of the dogs studied.*

In order to exclude the conversion of pregnenolone to dehydroepiandrosterone by the analytical procedures, 50 and 100 μg. pregnenolone were added to 10 ml. of a 5% albumin solution and subjected to the various steps of the method (14). No dehydroepiandrosterone could be determined. Likewise, the incubation of 10 ml. fresh whole dog blood or 10 ml. fresh dog plasma with 500 μg pregnenolone for 1 hour at 37° C failed to give evidence of a transformation of pregnenolone to dehydroepiandrosterone. Further, two 5 mg. samples of pregnenolone in propylene glycol and 2 samples containing 500 μg. of 17α-hydroxypregnenolone were as-

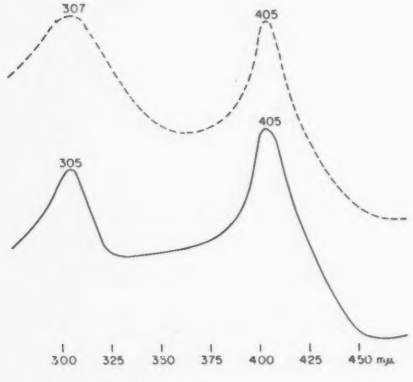

FIG. 1. Sulfuric acid spectrum of standard dehydroepiandrosterone and dehydroepiandrosterone obtained from the isolated dog leg after administration of pregnenolone

– – – – – Dehydroepiandrosterone from the isolated dog leg

———— Standard dehydroepiandrosterone

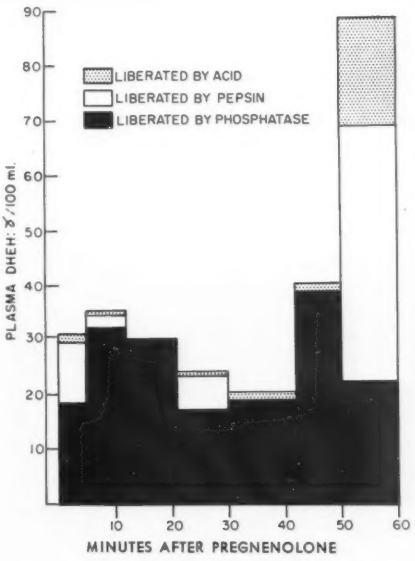

FIG. 2. Hydrolysis of DHEA conjugates in blood plasma from the isolated dog leg following the administration of 50 mg. pregnenolone. All levels are expressed in micrograms/100 ml.
Pepsin: obtained from Worthington Biochemical Corp., Freehold, N.J.
Phosphatase: obtained from Worthington Biochemical Corp., Freehold, N.J.

sayed for dehydroepiandrosterone by the analytical procedures (14). Dehydroepiandrosterone could not be detected in these samples. Finally, we were unable to detect any dehydroepiandrosterone in the randomly labeled pregnenolone used in this investigation.

DISCUSSION

The data of this paper demonstrate that

a) the adrenal secretion of dehydroepiandrosterone in "stressed" dogs is very minute compared to that of 17-hydroxycorticosteroids,
b) the concentration of dehydroepiandrosterone in the systemic blood of the dog is, by the best estimation, only equal to that of 17-hydroxycorticosteroids,
c) pregnenolone and 17α-hydroxypregnenolone can be converted to plasma dehydroepiandrosterone in normal dogs and dogs exposed to the "stress" of sodium pentobarbital anesthesia and surgical operation. In the absence of adrenals, testis and liver, a conversion of randomly labeled tritiated pregnenolone to tritiated plasma dehydroepiandrosterone still takes place.
d) tissues of the right hind leg in the dog with an intact arterial blood supply *can* convert pregnenolone and 17α-hydroxypregnenolone to plasma dehydroepiandrosterone.

Some of these findings warrant further comment. Hechter *et al.* (4) and Carstensen (5) have observed that 17-ketosteroids are secreted by the canine adrenal; however, in both investigations the 17-ketosteroid ma-

terial found also possessed a Δ^4-3-keto configuration. Our data will not allow the conclusion that Δ^5-3β-hydroxy-17-ketosteroids are secretory products of the canine adrenal cortex since in the adrenal plasma volumes processed (40–60 ml.) the concentration of dehydroepiandrosterone was too low to be identified adequately. Further, dehydroepiandrosterone was not found in adrenal vein blood of control animals or animals given pregnenolone unless the plasma had been subjected to hydrolysis. It is not very likely that the adrenal cortex secretes a conjugated steroid. Finally, when systemic blood and adrenal vein blood were obtained simultaneously and assayed for dehydroepiandrosterone, the systemic blood had the same concentration of this steroid as the adrenal vein blood.

These observations could permit the conclusion that a species difference exists between dog and man in secretion of C-19 steroids; however, opinion concerning the production of dehydroepiandrosterone by the human adrenal is still in a state of flux. The *in vitro* synthesis of dehydroepiandrosterone from sodium acetate-1C^{14} has been achieved by slices of an adrenal gland obtained from a patient suffering from the adrenogenital syndrome (24). "ACTH had no significant effect on the accumulation of dehydroepiandrosterone in this system" (24), whereas the administration of ACTH *in vivo* will increase plasma levels of dehydroepiandrosterone in normal human subjects (25). However, this ACTH response is delayed compared to the prompt response of plasma 17-hydroxycorticosteroids (25). From a study of steroid concentrations in adrenal vein blood and urine in a woman with virilism, Bush *et al.* (26) concluded that only about 10% of the dehydroepiandrosterone excreted could be accounted for in terms of its adrenocortical production, and Plantin *et al.* (27) isolated about 700 micrograms dehydroepiandrosterone from the adrenal glands of a virilized female patient excreting about 84 mg. dehydroepiandrosterone per 24 hours.

In the normal man, the plasma concentrations of dehydroepiandrosterone have been found to range between 40–70 μg./100 ml. (28), whereas free plasma 17-hydroxycorticosteroids are only about 25%–40% of that of dehydroepiandrosterone (29). In the normal dog from 2–4 μg. free 17-hydroxycorticosteroids are found in 100 ml. plasma (23) and the plasma concentration of dehydroepiandrosterone apparently does not exceed 3 μg./100 ml. (Table 2). The reason for this difference between man and dog is unknown, unless as suggested by Paschkis *et al.* (6), the dog rapidly metabolizes 17-ketosteroids to compounds not giving rise to a Zimmermann chromogen.

The chemical data presented will allow the conclusion that both pregnenolone and 17α-hydroxypregnenolone may serve as precursors for plasma dehydroepiandrosterone in the dog. Whether or not pregnenolone has to be hydroxylated in position 17 before being metabolized to dehydroepiandrosterone cannot be stated from the body of information pre-

sented. However, the recent isolation and identification of 17α-hydroxypregnenolone in the adrenal vein blood of dogs given ACTH (30) could suggest that 17α-hydroxypregnenolone secreted by the adrenal gland is a precursor for dehydroepiandrosterone in the systemic blood of the dog under physiological conditions.

Where is pregnenolone or 17α-hydroxypregnenolone converted to plasma dehydroepiandrosterone in the dog? In light of the data we are forced to propose the rather unattractive theory that this conversion *can* take place in the absence of adrenals, testis and liver. This does not necessarily mean that these organs are not involved in the metabolism of pregnenolone and 17α-hydroxypregnenolone to plasma dehydroepiandrosterone in the normal animal, but it would seem from these results that their contribution may be minor in the dog. What relative role the tissues of the hind leg of the dog play in the metabolism of 17α-hydroxypregnenolone to plasma dehydroepiandrosterone in the intact animal cannot be evaluated from our data since they are computed from anesthetized mongrel dogs differing in age, weight and nutritional status.

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ACTION OF ADRENALCORTICAL STEROIDS ON CULTURED CELLS

HENRY GROSSFELD

Department of Orthopedic Surgery, College of Physicians and Surgeons, Columbia University, New York, N. Y.

ABSTRACT

A correspondence exists between the direct action of adrenocortical hormones on cultured cells and their action in the organism. Inhibition of growth and depression of respiration and of production of mucopolysaccharide in tissue culture by hydrocortisone in high concentration appear to be related to the inhibition of wound healing by excess hydrocortisone. The growth-inhibitory action of meticortelone on cultured cells is, as in the whole organism, about 3 times greater than that of hydrocortisone. Hydrocortisone in low concentration stimulates respiration thus increasing energy production. In the organism, this appears to be the cause of the increase of work efficiency under the influence of hydrocortisone. In high concentrations, hydrocortisone depresses respiration and increases aerobic glycolysis. The present finding that excess hydrocortisone stimulates anaerobic metabolism provides a basis for analyzing the role of the adrenal cortex in the performance of strenuous work in states of emergency, for which a supply of anaerobic energy is required (24, 25).

INHIBITION of protein synthesis and stimulation of gluconeogenesis from amino acids by glucocorticoids are firmly established. Less conclusive evidence has been gained regarding the question whether glucocorticoids affect the rate of glucose oxidation. A decrease of glucose oxidation and impaired utilization of carbohydrate have been found when large doses of corticoids were given (1, 2). The "diabetogenic" effect of C-11-oxygenated steroids was accordingly thought to be caused not only by excessive gluconeogenesis from amino acids, but also by diminished glucose utilization (4–6). In recent work, however, in which labeled glucose was used, no significant impairment of oxidation under the influence of glucocorticoids was detected (2, 3). On the other hand, the stimulatory effect of glucocorticoids on the ability of muscle to do work indicated an increase of energy production, and in many reports the adrenal cortex was found largely responsible for stimulating metabolic rate (1, 7). It was thought, therefore, that an investigation into the direct action of glucocorticoids on cultured cells might contribute to the elucidation of this question.

METHODS

The method of growth estimation by enumeration of cell nuclei as described by Sanford *et al.* (8) was used for growth estimation of established cell strains. Earle's strain L

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derived from a fibroblast isolated from a culture of normal adipose subcutaneous tissue of the mouse was found very responsive to the action of hydrocortisone. This cell strain, however, like other established cell strains was not producing mucopolysaccharides *in vitro*, as were fibroblasts in primary cultures (9–11). The action of meticortelone, which in the intact organism is more potent than hydrocortisone, was compraed with the action of the latter.

To primary cultures of fibroblasts growing from explants of embryonic chick bone in roller tubes or in Petroff flasks and regularly producing hyaluronic acid *in vitro* for several months (10), hydrocortisone was added in various concentrations for studying the effect of hydrocortisone on production of acid mucopolysaccharide.

For analyzing the effect of hydrocortisone, in low concentrations, on survival of starved cells, the following procedure was employed. To the medium of cover-slip cultures consisting of plasma and chick amniotic fluid (12), hydrocortisone in various concentrations (0.10–20 μg./ml.) was added. After incubation for various periods of time without renewal of medium, vital staining with neutral red was carried out, and 210 cultures of fibroblasts used in the experiment, including control cultures without hydrocortisone, were classified in groups A (all cells alive), A1 (most cells alive), B (all cells dead) and B1 (most cells dead).

The action of hydrocortisone, and of desoxycorticosterone on respiration and aerobic glycolysis of cultured cells was investigated. Strain L cells were carried in T-60 flasks in a medium of embryo extract, horse serum and Earle's balanced salt solution in proportions of 20:40:40, containing 0.22% $NaHCO_3$, 0.0125% $NaH_2PO_4 \cdot H_2O$, and phenol red as pH indicator; the pH was adjusted to 7.40. The medium was changed 2 to 3 times a week. Cells of 8 flasks, each containing about 15×10^6 cells, were pooled, centrifuged, and the residue suspended in 25 ml of fresh medium. Three ml of the cell suspension were put into each of the 8 Warburg flasks, and the remainder was used for enumeration of cell nuclei. To each of four Warburg flasks 0.50 ml of Earle's salt solution containing hydrocortisone in a concentration of 125 μg. per ml was added, and to 4 control flasks, 0.50 ml. Earle's salt solution without hydrocortisone. The stock solution of hydrocortisone contained 50% ethanol. Therefore, ethanol in equal final dilution was added to the control vessels. In most cases control and experimental vessels were in duplicate: two control vessels containing 0.20 ml. of 15% KOH in the center well to trap carbon dioxide, and two control flasks containing 0.20 ml. H_2O in the center well for measurement of aerobic glycolysis; two flasks for O_2-uptake in the presence of hydrocortisone and two for aerobic glycolysis in the presence of hydrocortisone. The manometer reading of the flasks with H_2O in the center well is the resultant of both the consumption of O_2 (as shown in the flasks with alkali) and the liberation of CO_2. Aerobic glycolysis is thus obtained by the difference between the readings on the flasks with and without alkali, assuming that the R.Q. is in the neighborhood of 1. This "direct method" (13) was employed because, for comparison of values obtained in the presence and in the absence of hydrocortisone, the effect, if any, of CO_2 on the rate of respiration, was assumed to be negligible. In a second group of experiments, to 4 of 8 T-60 flasks containing equal numbers of cells, hydrocortisone in concentrations of 0.50 to 1.50 μg./ml. was added at the time of explantation. After 72 hrs., hydrocortisone in the same concentration was added to each of the 4 flasks (without renewal of the medium) plus 2.50 ml. new medium. After further 24 hrs. (96 hrs. from the beginning of the experiment), hydrocortisone in the same final concentration was again added to cell suspensions of the 4 Warburg flasks, before putting them in the manometric bath. Each Warburg flask contained in its main compartment equal numbers of cells suspended in 3 ml. of new medium, to which 0.50 ml. Earle's salt solution containing hydrocortisone was added. Four control vessels were treated the same way, but without hydrocortisone. In 4 experiments, cholesterol,

TABLE 1. CELL GROWTH INHIBITION BY HYDROCORTISONE

Exper. no.	Conc. hydrocortisone μg./ml.	Number of cell nuclei × 10^6		Percent inhibition
		Control	Hydrocortisone	
1	125	2.44	1.33	46
2	125	0.75	0.45	40
3	125	0.99	0.55	45
4	125	0.69	0.33	52
5	125	1.53	0.92	40
6	125	1.46	1.04	29
7	125	0.71	0.46	35
8	125	1.81	1.15	36
9	125	0.95	0.68	28
10	125	1.56	0.91	42
11	125	1.68	1.01	40
12	50	2.62	2.32	11
13	200	2.45	1.02	58
14	25	2.45	2.43	21

cortisone acetate, and the mineralocorticoid desoxycorticosterone were also added as controls.

RESULTS

The average growth inhibition of strain L cells caused by a single dose of hydrocortisone in concentration of 125 μg./ml. was found to be 40% in 11 experiments (Table 1), while meticortelone was found to be much more inhibitory (Table 2).

Production of hyaluronic acid in primary cultures of tissues of mesenchymal origin was reduced, and after 1–2 renewals of medium it stopped altogether following treatment with high concentrations of hydrocortisone (about 150 μg./ml.).

Cultures in a medium of chicken plasma and amniotic fluid, treated with hydrocortisone in low concentrations and incubated for 8 days without change of medium showed a longer average time of survival, namely, 48% of cultures were classified as group A, 35% as group A1, none as group B, and 17% as group B1, while of control cultures 8% belonged to group A,

TABLE 2. ACTION OF METICORTELONE ON GROWTH ESTIMATED BY CELL NUCLEI ENUMERATION

Number of exper.	Concentration μg./ml.	Number of cell nuclei 10^6	Percent inhibition
1	0	2.5	—
	5	2.43	4
	12.5	1.67	36
2	0	0.80	—
	25	0.54	33
	100	0.40	50
3*	0	1.79	—
	5	1.44	19
	12.5	1.00	45

* Treated twice with meticortelone.

TABLE 3. ACTION OF A SINGLE GROWTH-INHIBITORY DOSE OF HYDROCORTISONE ON RESPIRATION AND AEROBIC GLYCOLYSIS

#	Concentration μg./ml.	μl. O_2 consumed after hours					μl. CO_2 evolved after hours*				
		1	2	3	4	5	1	2	3	4	5
1	150	− 81	−128	−144	−143	—	+42	+ 33	+28	+ 19	—
	0	−115	−166	−214	−221	—	0	0	0	0	—
2	125	− 19	− 28	− 47	—	—	+96	+185	+134	—	—
	0	− 44	− 70	− 98	—	—	+21	+ 50	+ 25	—	—
3	125	− 21	− 33	− 36	− 45	—	+68	+113	+153	+141	—
	0	− 40	− 57	− 84	− 97	—	+16	+ 40	+ 25	+ 17	—
4	125	− 55	−102	−130	−146	−153	+88	+ 40	+ 72	+ 92	+86
	0	− 88	−155	−225	−258	−280	0	0	0	0	0
5	125	− 13	− 50	− 65	− 62	− 73	+69	+110	+ 83	+ 65	+45
	0	− 41	−157	−240	−313	−362	0	0	0	0	
6	100	− 87	−141	−163	−188	—	+43	+131	+143	+123	—
	0	− 99	−175	−187	−210	—	0	0	0	0	—
7	60	− 54	− 72	−106	−113	—	+52	+138	+149	+169	—
	0	− 78	−110	−139	−149	—	0	0	0	0	—

* After subtraction of respiratory CO_2.

24% to A1, 47% to B, and 21% to B1. After 12 days of incubation, 28% of treated cultures were found in group A, 52% in A1, none in B, and 20% in B1, while in control cultures, 3% were found in group A, 24% in group A1, 50% in B, and 23% in B1. When, after 8 days of incubation, cultures were kept at room temperature for 12 more days a similar effect was observed.

The investigation of the influence of hydrocortisone on respiration and

TABLE 4. STIMULATION OF RESPIRATION BY LOWER CONCENTRATIONS OF HYDROCORTISONE

#	Concentration μ⁻/m'.*	μl. O_2 consumed after hours				
		1	2	3	4	5
1	1.50	−181	−276	−374	−474	−536
	0	−133	−190	−239	−271	−273
2	1.50	−193	−363	−492	−668	—
	0	−141	−241	−300	−346	—
3	1.50	−130	−219	−290	−360	—
	0	− 94	−166	−193	−226	—
4	2.00	−176	−379	−524	−664	—
	0	− 97	−227	−313	−346	—
5	0.50	− 78	−123	−150	−187	—
	0	− 67	− 96	−104	−114	—
6	1.25	− 72	−100	−129	−145	−152
	0	− 47	− 60	− 64	− 81	− 76

* Added to the cultures 3 times.

TABLE 5. ACTION OF CORTICOSTEROIDS ON RESPIRATION OF STRAIN L FIBROBLASTS

Steroid	Concentration μg./ml.*	μl. O_2 consumed after hours			
		1	2	3	4
Hydrocortisone	1.25	−128	−217	−269	−327
Desoxycorticosterone	1.25	− 86	−137	−158	−174
Control	—	− 78	−115	−121	−125
Cholesterol	1.25	− 93	−136	−126	−128

* Added to the cultures 3 times: 96 hours, 24 hours, and 1 hour before the experiment in Warburg's manometric bath.

on aerobic glycolysis of cultured cells gave the following results. In the first group of experiments, a single dose of hydrocortisone in high concentration added to the cell suspension in Warburg flasks caused inhibition of respiration coupled with significant increase of aerobic glycolysis (Table 3). Hydrocortisone in the same concentration added in a single dose to cultured cells cause growth inhibition averaging 40% for 125 μg./ml. as determined by enumeration of cell nuclei (Table 1). In the second group of experiments, hydrocortisone added to the cultures at 96 hrs., at 24 hrs., and immediately before the manometric experiment, caused stimulation of respiration (Table 4). The mineralocorticoid desoxycorticosterone showed a considerably smaller effect than hydrocortisone (Tables 5–6).

In cultures treated with a single dose of high concentrations of hydrocortisone, the average size of nucleoli was estimated to be smaller than in untreated cultures. Diminution in size of nucleoli was thought to be related to depression of protein synthesis by hydrocortisone in high concentrations (16).

DISCUSSION

The original manometric Warburg procedure for determination of glycolysis rates has been used in the present work in which the lactic acid

TABLE 6. ACTION OF CORTICOSTEROIDS ON RESPIRATION OF STRAIN L FIBROBLASTS

Steroid	Concentration μg./ml.*	μl. O_2 consumed after minutes				
		30	90	150	210	240
Hydrocortisone	1.25	−84	−253	−358	−419	−439
Desoxycorticosterone	1.25	−62	−159	−223	−219	−231
Cortisone	1.25	−64	−151	−176	−187	−195
Control	—	−56	− 87	− 84	− 88	− 93

* Added to cultures 3 times: 96 hours, 24 hours, and 1 hour before the experiment in Warburg's monometric bath.

produced was measured indirectly by estimation of the cubic millimeters of CO_2 liberated by lactic acid from bicarbonate and the assumption has been made that the respiratory quotient is in the neighborhood of 1 (14, 15).

Results of measurements of aerobic glycolysis by chemical estimation of lactic acid, which are in substantial agreement with the present findings, have been reported (17, 18). The action of an adrenocortical extract, standardized to contain the equivalent of 1 mg. of hydrocortisone per ml. on thymus lymphocytes and tissue slices from various organs was studied. Aerobic glycolysis was increased independently of the action on respiration, and respiration was inhibited only by high concentrations of the extract. The effect could be obtained only when intact cells were used. The action on respiration and aerobic glycolysis was correlated with the lymphocytolytic effect of the same preparation. This effect, however, could not be found when, instead of adrenocortical extract containing hydrocortisone, hydrocortisone alone was used. The discrepancy appears to be caused by the difference in the methods of preparing the soluble hydrocortisone (19).

In many instances, the optimal steroid concentration for production of hormonal effects in *in vitro* experiments has not been determined. There is, however, no indication that specific effects of steroids *in vitro* can be achieved by the same concentrations as those which are known to act in the organism. Many results of *in vitro* experiments on tissue slices as well as in tissue culture suggest that specific effects are obtained *in vitro* with steroid concentrations which are higher than those required for obtaining similar effects in the organism. It is possible that in *in vitro* experiments, some factors are lacking which in the organism are instrumental in concentrating hormones on specific target cells. In the absence of these factors, the added steroid hormone might be equally absorbed by all of the cells and the effective concentration might thus need to be higher *in vitro* than in the intact organism in which only target cells are affected.

About 7 times as much glucose is necessary to produce a given quantity of ATP when glycolysis instead of respiration is used for energy production (20).[1] At the same time, 6 to 7 times more glucose was found to be synthesized in animals treated with excess glucocorticoids than in control animals (4). This striking agreement is consistent with the present finding that in the presence of excess hydrocortisone a shift towards a more anaerobic steady state may take place, in which 7 times more glucose has to be made available.

The present findings may provide a basis for interpretation of a number of data obtained *in vivo* and *in vitro* experiments which might appear con-

[1] Facultative anaerobic earthworms (21), and many other types of worms (22) consume up to 6 times as much glycogen in fermentation as in aerobiosis. The same ratio was found to hold for frog muscle (23).

tradictory. On the one hand, the mechanism of action of adrenal cortical steroids appears to be one influencing normal function and survival of the intact cell. This is clearly seen in adrenalectomized "adynamic" animals as well as in Addison's disease, where survival depends on treatment with corticosteroids, and likewise in tissue culture, where hydrocortisone may promote survival of starved cells. It is clearly seen in the striking effect of adrenocortical hormones on the ability of muscle to do work. On the other hand, hydrocortisone inhibits cellular growth, and it may also lead to the disruption of cellular structure with the release of cell constituents, for instance, the destruction of lymphatic tissue and of thymus following excessive corticoid action.

The present findings suggest that hydrocortisone in lower concentrations, by stimulating respiration increases energy production and work efficiency. In conditions of hydrocortisone, excess sugar is synthesized from amino acids. At the same time, proteolysis, which is consistently accelerated in proportion to the degree of corticoid excess, may lead to impairment and damage of cellular stores of certain target cells, especially when not only storage protein but also structural protein is depleted.

The energy for increased muscle tone stimulated by epinephrine could be derived from increased respiration stimulated by glucocorticoids in low concentration.

The present findings may have some bearing on the question of the role of the adrenal cortex in certain states of emergency. The muscle suddenly put under strain has been found to rely predominantly on a supply of anaerobic energy (24, 25). If oxidative breakdown of sugar for some reason becomes insufficient for quick supply of energy, the latter may temporarily be replaced by glycolysis (20). Glucocorticoids, when in excess of physiological levels, appears to be instrumental in the shift to anaerobic metabolism and to the production of excess glucose from amino acids which is necessary when energy is produced anaerobically. The shift may be temporary and, when oxidative metabolism is restored, the utilization of sugar can be reduced to the original $\frac{1}{7}$ part required for production of the same amount of energy by oxidative breakdown of sugar. Anaerobic metabolism has been found necessary for the initiation of unusually strenuous work (24, 25). Since anaerobic metabolism is stimulated by an excess of hydrocortisone, the role of the adrenal cortex in "emergency work" can now be more successfully analyzed.

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A COMPLEMENTARY ACTION OF CORTICOSTERONE AND DEHYDROEPIANDROSTERONE ON THE MOUSE ADRENAL, WITH OBSERVATIONS ON THE REACTIVITY OF REPRODUCTIVE TRACT STRUCTURES TO DEHYDROEPIANDROSTERONE AND 11-HYDROXY-ANDROSTENEDIONE[1]

EVELYN HOWARD

Department of Physiology, Johns Hopkins University School of Medicine, Baltimore, Maryland

ABSTRACT

Dehydroepiandrosterone (DHA) and corticosterone (B) had complementary and roughly additive effects in reducing adrenal weights in mice when the adrenal X zone was present. DHA suppressed the X zone whereas B reduced the size of the zona fasciculata. DHA had no effect on adrenal weights in mature mice in which the X zone was not present. The suppression of the X zone by DHA was compatible with apparently normal ovarian and uterine maturation.

X zone suppression was produced by doses of DHA which gave only minimal stimulation to seminal vesicle growth, but had pronounced effects on the growth of the preputial glands and the phallus in either sex. 11-hydroxy-Δ^4-androstenedione was somewhat less active than DHA in suppressing the X zone, but its effects on reproductive tract structures were of the same order of magnitude. If the reactivity of the human embryonic phallus is similar to that of the immature rodent, excessive production of either of these two adrenal androgens could be expected to account for the genital abnormalities in human female infants with adrenal hyperplasia.

DEHYDROEPIANDROSTERONE (Δ^5-androstene-3β-ol-17-one, or DHA) has been found to be a major component of plasma 17-ketosteroids in peripheral human blood (1) and has been identified in human fetal adrenals (2). Furthermore, DHA appears relatively upstream in the androgen metabolic sequences, inasmuch as the double bond is not known to move from the C_{4-5} position to the C_{5-6} position *in vivo* (3). 11-hydroxy-Δ^4-androstene-3,17-dione (11-OH-Δ^4-dione) has also been identified in human fetal adrenals (2) and in adrenal venous blood of various species (4, 5). These observations point to the desirability of more study of the physiological effects of these steroids than has appeared hitherto.

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DHA has been characterized as having about 1 to 2% of the activity of testosterone on the male accessory glands of castrated rats (6, 7). However, the usual biological tests for androgenic activity were developed in the course of studies on testicular hormones, so that the performance of the adrenal C_{19} steroids in these tests may not give us much insight into their physiological significance. The renotrophic activity of DHA was 9% of that of testosterone (7). DHA had an appreciable activity in stimulating the growth of certain shoulder muscles of the guinea pig, but had much less activity than testosterone on muscles such as the temporal and masseter which had a relatively large response to testosterone (8).

In ovariectomized rats, DHA produced only a slight increase in uterine weight (6). No potentiation of the effects of estrone by DHA was detected, either in ovariectomized rats (6) or in immature mice (9). Nevertheless a considerable uterine and vaginal stimulation by DHA in intact rats (10) has been reported, but has not been satisfactorily explained.

The X zone of the mouse adrenal is known to be suppressed by doses of Δ^5-androstenediol which do not stimulate the growth of the seminal vesicles (11). Since adrenalectomy does not appear to influence the state of the seminal vesicles in castrated mice (12), the properties of steroids which suppress the X zone without stimulating the vesicles are of interest.

MATERIAL AND METHODS

White mice of Swiss stocks were used. Animals were housed in an air conditioned room maintained at 22–24° C. Operations were performed under ether anesthesia. In so far as possible, litter mates always controlled each other: i.e., females of a given litter were divided at random among the various categories, as, a) untreated control, b) DHA, c) corticosterone, etc. At autopsy, animals were killed with ether, and, with the aid of a dissecting microscope, tissues were dissected free of adnexa under saline, blotted to remove excess moisture and weighed quickly on a torsion balance. They were then fixed in Bouin's fluid for histological examination. The phallus was isolated after removing the integument. Seminal vesicles were weighed with their contained secretion, by transecting the ducts close to the urethra. If the uteri contained secretion, it was removed by blotting with gauze.

Hormones were administered as pellets of 3 to 9 mg. initial weight, depending on the size of the mouse. The rate of absorption was determined by noting the change in pellet weight. The initial rate of solution may be more rapid than it becomes later, but in the case of DHA the rate of absorption for the first 7 days did not differ appreciably for the rate for periods up to 30 days. Single pellets in adults lost weight at a rate of 0.16 mg./day (range 0.13–0.19); in 12 to 30 day old mice at 0.14 (0.11–0.17); and in newborn at 0.12 (0.09–0.16). Corticosterone was more soluble, and was absorbed at a rate of 0.38 mg./day (0.31–0.46). For the first 5 to 7 days after implanting a 5–6 mg. corticosterone pellet, there was a very marked interference with body growth, and thereafter a gradual resumption of growth, suggesting that in this case the initial rate of steroid absorption might be greater than it became later. Corticosterone was not reimplanted during the experimental periods used because of the severe reaction that it caused. In some cases the pellet of corticosterone was presumably completely absorbed several days prior to autopsy. DHA pellets of considerable size were usually present at autopsy. Pregnenolone (Δ^5-pregnene-3β-ol-20-one) was less rapidly absorbed, at a rate of only 0.014 mg./day.

The most rapidly absorbed steroid used in this study was 11-hydroxy-Δ^4-androstene-3,17-dione, which lost weight at a rate of 0.5 mg./day, and was accordingly implanted more than once during a test period.

EXPERIMENTS AND RESULTS

A. The influence of steroids on the adrenal. A study has been made of the effects of DHA on the adrenal when the androgen was administered either alone or in combination with corticosterone (B). The latter steroid was selected since it appears to be the chief glucocorticoid produced by the mouse adrenal (13, 14).

TABLE 1. INFLUENCE OF DEHYDROEPIANDROSTERONE (DHA) AND 11-HYDROXYANDROSTENEDIONE (11-OXY) ON ADRENAL AND PREPUTIAL GLAND WEIGHTS IN MALE MICE

Steroid and dose mg./day	Status[1]	Body wt. gms. Means ± S.E.	Preputials mg./10 gm. b.w. Means ± S.E.	Adrenals mg./10 gm. b.w. Means ± S.E.
Experiment I				
DHA 0.14	Castrate (9)	27.4 ± .86''	12.2 ± 2.3*	2.05 ± .087*
None —	Castrate (7)	26.6 ± .70*	3.9 ± 0.2	2.71 ± .165
None —	Intact (5)	31.4 ± .56	29.3 ± 3.1	1.55 ± .065
Experiment II				
11-oxy 0.34	Castrate (4)	25.3 ± .9	8.5 ± .95''	1.98 ± .03'
None —	Castrate (5)	23.6 ± .9	4.4 ± .54	2.72 ± .23
Experiment III				
DHA 0.16	Intact (10)	33.9	—	1.21 ± .08
None —	Intact (10)	33.8	—	1.17 ± .05

[1] Numbers of mice in ( ). P values are indicated as follows: *, P = .01 or less; '' .02–.03; ' .05. Body weights in Experiment I are compared with the intact group, otherwise comparisons are between untreated and treated castrates.

Data on males are presented in Table 1. In Experiment I, the mice were castrated at 4 days of age and implanted with pellets of DHA at 12 or 22 days of age: in Experiment II the animals were castrated at 12 days of age and immediately implanted with pellets of 11-β-hydroxy-Δ^4-androstene-3,17-dione, which were renewed after two weeks. Both groups were autopsied at 6 weeks of age. The intact males of Experiment III were 3 to 10 months of age when pellets were implanted, controls and treated being matched as to age, and were autopsied after 4 weeks of treatment.

Treatment of the immature females was initiated at either 12 days of age or at 18–25 days, and the animals were autopsied at 41 ± 3 days. Since no significant differences in adrenal condition were detected relative to these age differences, the findings are combined in Table 2. Body weights at 12 days of age are of the order of 5 gms., while at 41 days body weights are of the order of 20 gms. or more, hence the steroid treatment covered a large part of the growth period. In Experiments I, II, and III, an ovulation, presumably the first, frequently occurred shortly before the time of

FIGS. 1 through 4. Mouse adrenal glands, from females 6 weeks of age, showing effects of corticosterone and dehydroepiandrosterone, singly and in combination. Conditions as given in Table 2. Magnification, 50×, Azan stain.

FIG. 1. Untreated control. Note the deeply stained X zone of moderate size which

autopsy. At this age the X zone would normally have achieved its maximum size. The adult females of Experiment IV were not treated until at least 6 weeks after delivery of a litter. They received DHA for 4 weeks.

There was a significant reduction in adrenal weight in both the castrated males (Table 1) and the immature females (Table 2) treated with DHA, but this steroid produced no significant change in adrenal weight in either the adult intact males or the parous females, which are normally without adrenal X zone. It is of interest that DHA did not, however, reduce adrenal weights in the castrated males to the size of the adrenals in the intact males. Furthermore the body weights in the castrates were not materially improved by DHA and remained significantly less than in the intact males. The 11-hydroxy-androstenedione was studied only in castrated males, where it produced a significant decrease in adrenal weight.

Histological examination of these adrenals showed that the weight reduction produced by DHA was associated with a marked reduction of the adrenal X zone, as illustrated in Figures 3 and 7. In the immature females on DHA the X zone was absent in most cases: in a few individuals it was represented by some small groups of cells. In the castrated males on DHA the X zone was usually represented by only a few perimedullary cells, in contrast to the litter mate castrated controls in which this zone was very well developed. In two castrated males in which the DHA pellet had been absorbed prior to autopsy considerable amounts of apparently regenerated X zone were present. Suppression of the X zone was incomplete with 11-hydroxy-androstenedione. Although the zone was considerably reduced in size in comparison with the untreated castrated controls, even with the larger dose of the 11-hydroxy compound the X zone was not as much suppressed in any individual as it was in most cases with DHA.

The adrenals of the castrated males and immature females given DHA assumed the characteristic mature structure. On the other hand, there was no evident change in the appearance of the adrenals in the mature males and females treated with DHA. Thus it appears that the effects of DHA on the mouse adrenal are primarily on the X zone.

It has been shown that the X zone appears to be dependent on a pituitary trophic factor, other than ACTH, present in gonadotrophic pituitary extracts (15). Hence DHA is presumably acting to suppress a pituitary

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contrasts with the more lightly stained definitive cortex, and the light central medulla.

FIG. 2. Corticosterone has produced a reduction in the weight of the adrenal but the X zone has not been suppressed. From a litter mate of specimen shown in Fig. 4.

FIG. 3. Dehydroepiandrosterone has produced a complete suppression of the X zone, and the gland has assumed its typical mature structure.

FIG. 4. Combined treatment with corticosterone and dehydroepiandrosterone has resulted in a reduction of the cortex to a narrow band of small crowded cells. The entire central light area is medulla.

TABLE 2. INFLUENCE OF STEROIDS ON ORGAN WEIGHTS IN INTACT FEMALE MICE

Steroid[1]	Body weight[2] at autopsy gm.	Ovaries[2] mg./10 gm. body wt.	Uterus[2] mg./10 gm. body wt.	Preputials[2] mg./10 gm. body wt.	Adrenals[2] mg./10 gm. body wt.
Experiment I. Immature					
DHA (6)	19.4 ± .50	2.77 ± .25#	35.5 ± 4.3	10.3 ± .73**	2.54 ± .06**
B (8)	17.2 ± .90′	3.24 ± .31	19.5 ± 4.1	2.6 ± .19	2.86 ± .14*
B+DHA (5)	17.2 ± 1.12	2.90 ± .14	35.9 ± 3.9	6.3 ± 1.21″	1.32 ± .13**
None (10)	19.5 ± .49	3.41 ± .15	35.8 ± 6.7	2.4 ± .22	3.68 ± .13
Experiment II. Immature					
DHA (8)	24.7 ± .87	3.28 ± .19	29.0 ± 3.6	7.2 ± 1.17″	2.12 ± .07**
B (8)	22.7 ± .91′	2.85 ± .30	31.8 ± 7.1	2.4 ± .17	2.32 ± .13*
B+DHA (10)	22.6 ± .90″	2.94 ± .14	35.5 ± 5.9	7.0 ± 1.10″	1.76 ± .11**
None (11)	25.1 ± .43	3.18 ± .26	34.3 ± 5.9	3.2 ± .46	3.18 ± .14
Experiment III. Immature					
DHA+Preg. (2)	20.9	—	35.2	12.7 ± 1.01	2.55 ± .16
B+Preg. (3)	16.1	—	36.8 ± 16.2	3.8 ± .59	2.71 ± .02*
B (3)	16.9	—	32.2 ± 10.1	3.3 ± .16	2.63 ± .13*
B+DHA (5)	17.1	—	38.4 ± 5.0	9.1 ± .90	1.19 ± .06
Experiment IV. Parous Adults					
DHA (5)	31.4	—	—	—	2.34 ± .15
None (5)	30.8	—	—	—	2.30 ± .18

[1] Number in parenthesis is number of mice.
[2] Mean values ± standard error.
P values are indicated as follows. **, P = .001 or less; *, .01 or less; ″, .02–.03; ′, .05 to .06; #, .08; relative to untreated controls for the respective groups, except for adrenals in group III which were compared with B+DHA.
Steroid designations: DHA, dehydroepiandrosterone; B, corticosterone; Preg., Δ^5-pregnene-3β-ol-20-one. Dosage in methods section of text.

hormone, although the steroid may also have some direct action on the adrenal, as does testosterone (16).

The effect of DHA on the adrenals of immature females is strikingly different from the effect of corticosterone (B), which is illustrated in Figures 2 and 6. Although B and DHA both produce a reduction in adrenal weights of the same order of magnitude (Table 2), the histological findings are quite different. It can be seen that B does not suppress the X zone, but exerts its effects primarily on the zona fasciculata, as might be anticipated from the recognized effectiveness of the glucocorticoids in suppressing ACTH. Furthermore, the effect on the adrenal of the combined administration of both B and DHA is roughly additive, as evidenced both by the weight reductions and by the histological findings. As illustrated in Figures 4 and 8, the combination of the two steroids reduces the adrenal cortex to a narrow area of small crowded cells with minimal amounts of cytoplasm and little evidence of zonation. Thus it appears that B and

DHA have a mutually complementary effect on the mouse adrenal, in that they each suppress primarily a different part of the cortex, presumably by inhibiting different pituitary trophic hormones.

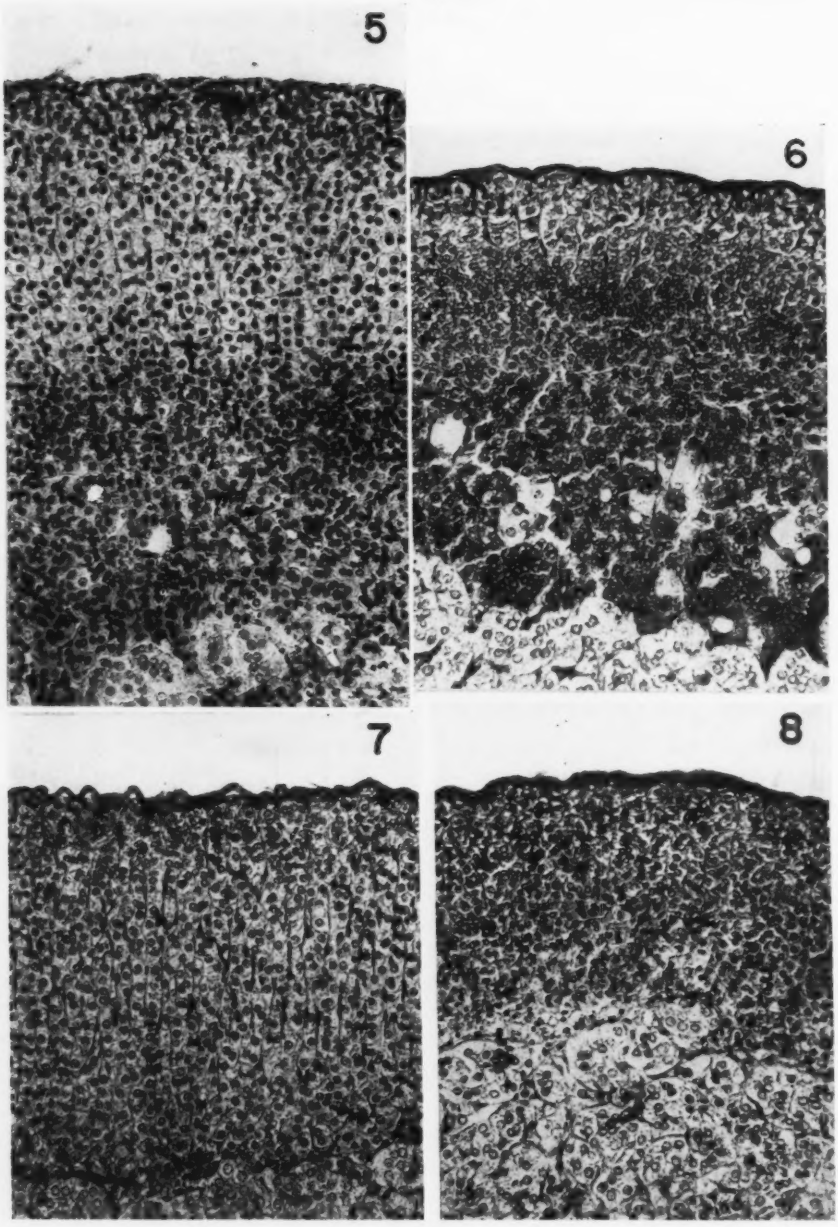
In mice given B alone, the cells of the outer part of the cortex frequently exhibited some differences in degree of regression. For example, in Figure 6 a zone of cells with relatively abundant lightly stained cytoplasm is evident a little beneath the outermost cells. In the mouse with the techniques used the demarcation between the glomerulosa and the fasciculata is indeterminate. Hence to interpret Figure 6 as indicating that the cells of the glomerulosa are relatively insensitive to the suppressing effects of corticosterone would be somewhat arbitrary, although attractive. Furthermore, the material was rather variable in the degree to which this apparent independence of the glomerulosa was manifest. However, in mice given both B and DHA there was a greater uniformity in the size of the cortical cells, as illustrated in Figure 8. This suggests that DHA treatment suppresses a factor which may have some degree of trophic influence on the outer parts of the cortex, as well as being essential for the maintenance of the X zone. The uniform cell size after the combined treatment with the two steroids is comparable to that reported after hypophysectomy in rats when actual cell measurements were made (17a, b).

Cortisone has been shown to reduce adrenal weight without suppression of the X zone in mice when given for 9 days at a dose of 1.3 mg. per day (18). This treatment reduced the size of the fasciculata cells and diminished body growth. Sakiz (19, 20) had reported that cortisone, while causing a reduction in the size of the fasciculata cells, produced an apparent hypertrophy of the X zone in castrated mice, in doses up to 10 mg. per day.

The implantation of pellets of pregnenolone (Δ^5-pregnene-3β-ol-20-one) in combination with either DHA or B resulted in no further modification of the adrenal of immature females beyond what was produced by DHA or B alone. However pregnenolone pellets were absorbed relatively slowly, at rates between 0.01 and 0.02 mg. per day.

Not only does corticosterone not suppress the X zone, but mice which had been treated with B showed a retardation of X zone degeneration as compared with untreated controls. Thus, of 11 litter mate pairs in which the controls exhibited a beginning or moderately advanced degenerative vacuolation in the X zone, the litter mates given B showed either significantly less degenerative vacuolation, or none at all. This effect may be the converse of the stimulation of X zone regression produced by exogenous ACTH (21).

The ovaries did not show much evidence of the absence of gonadotrophins in the young females on DHA. Although there was a moderate reduction in ovarian weights on DHA in group I, this effect was not mani-



FIGS. 5 through 8. Higher magnification (200×) of areas from glands shown in figures 1 to 4.

fested in group II.[2] Corpora lutea were present in the ovaries of 66% of the mice on DHA, an incidence not essentially different from that in the ovaries of the untreated controls. Hence, if DHA is acting to suppress a factor trophic for the X zone this is not incompatible with a fairly normal ovarian maturation.

In order to see whether the X zone suppression produced by DHA in the intact females could be secondary to a stimulation of androgen production by the ovary, an examination was made of the effects of DHA in ovariectomized females. Operations were performed at 11–13 days of age, and pellets of DHA implanted at the time of operation. At autopsy at 6 weeks of age, the adrenals of four DHA treated mice were without X zone, while well developed X zones were present in the ovariectomized controls. The adrenal:body weight ratios were reduced 24% by DHA treatment after ovariectomy. Hence X zone suppression by DHA is not dependent on the presence of the ovaries.

In the mice of Experiment I, Table 2, the relative uterine weights were somewhat reduced by corticosterone treatment in comparison with the untreated controls. However, DHA alone produced no uterine weight modification, further evidence that the dosage of DHA which was sufficient to suppress the X zone did not materially interfere with ovarian-uterine maturation. DHA in combination with B appeared to block the inhibition of uterine growth produced by B alone. An antagonism between estradiol and corticoids with respect to uterine metabolism and growth has been demonstrated by Szego and Roberts (22) and by Velardo and Sturgis (23). It is possible that the reduction in uterine growth in B-treated mice of Experiment I is an expression of this effect. However these experiments were not designed to examine the question of steroid interactions in the uterus, and it is possible that the smaller uterine size in the B-treated mice of Experiment I might reflect merely a retardation of ovarian maturation secondary to a negative N balance. Furthermore, B inhibition of uterine growth was not evident in Experiments II and III.

[2] The mice used in Experiment I, Table 2, were from a different source than those used in the other experiments of this table.

FIG. 5. Untreated control. The definitive cortex in the upper part of the photograph is roughly equal in width to X zone in the lower part. There are a few groups of very light medullary cells in the lower part of the photograph.

FIG. 6. Corticosterone treatment. There has been a reduction in the size of the cells particularly in the region of the zona fasciculata but not in the glomerulosa. The X zone nuclei seem somewhat enlarged.

FIG. 7. Dehydroepiandrosterone treatment. A typical adult structure without visible X zone.

FIG. 8. Combined treatment with corticosterone and dehydroepiandrosterone has resulted in a very narrow cortex composed of cells of nearly uniform size, with little cytoplasm.

B. The influence of dehydroepiandrosterone and 11-β-hydroxy-Δ⁴-androstenedione on the growth of the seminal vesicles, preputial glands and phallus. Previous studies have shown that, although DHA had a potency only 1 to 2% of that of testosterone in stimulating the growth of the seminal vesicles in castrated rats (6) and mice (7), it was at least as effective as testosterone in promoting the growth of the preputial gland in castrated male rats (24). In hypophysectomized female rats, DHA was about half as potent as testosterone on the preputial glands (25).

Previous observations on the biological properties of 11-hydroxy-Δ⁴-androstenedione are not known to the author. Δ⁴-androstene-3,11,17-trione has a moderate activity on the chick comb comparable to that of DHA or androstenedione (26). 11-β-hydroxy-androsterone was one-fourth as active as androsterone on the chick comb (27). Tests of the anabolic effectiveness of a number of C_{19} steroids in nitrogen balance studies in humans indicated that 11-β-hydroxylation results in loss of activity (28).

In the present study it has been observed that the preputial glands of castrated male mice (Table 1) and intact females (Table 2) are responsive to DHA and to 11-OH-Δ⁴-dione. The potency of the latter was less than that of the former: over twice the dose of 11-OH-Δ⁴dione produced, if anything, less effect than did DHA. It is of interest that corticosterone showed no tendency to cause preputial enlargement, and only slightly interfered with the effects of DHA on preputial size (Table 2).

In the course of a search for tissues which might be especially reactive to adrenal androgens it became apparent that the phallus is considerably more reactive than are the seminal vesicles. Phallic growth in the castrated male rat (24) and in the female rat (10) is known to be stimulated by DHA, but the effect has received little quantitative study. Phallic growth has been seldom used in biological assays, but it was found that phallic weights were quite uniform. In Experiment I, Table 3, the ratio of the standard errors of the means to the respective means averaged 0.08 for the seminal vesicle and 0.04 for the phallic-body weight ratios. In Table 3 the growth of the phallus is compared with that of the seminal vesicles in the same animals. The percentage increment in organ weight is calculated as the observed weight minus the untreated castrate weight divided by the intact weight minus the untreated castrate weight, using the organ-body weight ratios in all cases. The phallic increments vary with the age at the time treatment was initiated, but in all cases the phallic increment is much greater than the vesicle increment (Table 3). In the older mice of Experiment III the vesicles were so minute that they were not weighed. The moderate increase in vesicle size produced by DHA in Experiment I (Table 3) was associated with greater complexity of acinar structure, but the vesicle epithelium did not show the cytological response characteristic of the fully developed effect of testosterone.

Korenchevsky *et al.* (29) observed that phallic growth was proportional

TABLE 3. TARGET ORGAN REACTIVITIES TO DEHYDROEPIANDROSTERONE AND 11-β-HYDROXY-Δ4-ANDROSTENE-3,17-DIONE

Status	Dose Mg./day	Dose Mg./10 gm. initial[1]	Vesicle wt. mg./10 gm. body wt. means ± S.E.	Vesicle increment[1] % of intact	Phallic wt. mg./10 gm. body wt. means ± S.E.	Phallic increment[1] % of intact
Experiment I: Mice						
C-4 (8)	—	—	0.52 ± .054	0.0	2.14 ± .07	0.0
C-4: DHA-4 (6)	0.12	0.44	1.62 ± .067*	3.0	7.92 ± .36**	47.3
C-4: DHA-12 (4)	0.14	0.26	1.36 ± .103*	2.3	6.36 ± .33**	34.6
C-4: DHA-22 (5)	0.14	0.12	0.83 ± .086″	0.9	3.07 ± .18*	7.6
Intact (8)	—	—	36.8 ± 2.9	100.0	14.34 ± .37	100.0
Experiment II: Mice						
C-12 (5)	—	—	0.92 ± .12	0.0	4.43 ± .14	0.0
C-12: 11 OH-12 (4)	0.34	0.63	2.77 ± .34*	5.1	10.99 ± .46**	66.2
Experiment III: Mice						
C-4 (9)	—	—	—	—	1.85 ± .06	0.0
C-4. DHA-92 (3)	1.5	0.37	—	—	4.95 ± .48″	25.4
Rats (Calculated from data of Korenchevsky *et al.*, 24)						
DHA	1	—	—	2	—	23
Testosterone	1	—	—	62	—	102
Testosterone	0.1	—	—	6.4	—	44

Abbreviations: C = castration at age, in days, given by the suffix. DHA = subcutaneous implantation of a pellet of dehydroepiandrosterone at age, in days, given by suffix. 11-OH = subcutaneous implantation of a pellet of 11-β-hydroxy-Δ4-androstene-3,17-dione. Numbers of mice in ( ). Mice of Experiments I and II were autopsied at 6 weeks of age, and those of Experiment III at 17 weeks of age. P values are indicated as follows. ** 0.001 or less, * 0.01 or less, ″less than 0.05.

[1] See text for explanation.

to androgen dose in immature castrated rats, and this we find to be true for D HA in a few observations. Thus, in three month old mice castrated at 4 days of age, treatment with DHA for 4 weeks at doses of 4.4, 4.0 and 2.8 mg./100 gm. body weight per day produced phallic-body weight ratios of 5.6, 5.2 and 4.0 mg./10 gm.

Since the steroids were administered as pellets, the dose could not easily be adjusted to the changing body size in growing animals. In Table 3 are given the initial hormone doses per 10 gms. body weight, calculated from the overall pellet weight losses. These initially different doses gradually approached a uniform value as growth progressed toward mature body size at autopsy. Some of the observed differences in the organ weights must be attributed to the initial differences in steroid dose. However, comparing those treated initially at 22 days with those treated initially at 12 days, it may be seen that the initial dose ratio was .12/.26 or 0.46 while the phallic increment declined as 7.6/34.6 or 0.22. Similarly, comparing the 12 and the 92 day old groups, it may be seen that the dose was uniformly greater in the

older mice but the phallic response was less. Hence it may be concluded that there is some degree of actual loss in phallic reactivity with increasing time after castration.

Although the experimental conditions in Experiment II, Table 3, were not identical with those of Experiment I, it may be concluded that the reactivity of the phallus to 11-hydroxy-Δ^4-dione is of the same order of magnitude as it is to DHA, and that the relative reactivities of the phallus and seminal vesicles are quite similar with the two compounds.

In Experiment I, the phallic increment in the two younger groups of mice was about 15 times the vesicle increment. A similar differential response to DHA in rats was calculated from data in the literature (24), and in included in Table 3 for comparison. In contrast to DHA, testosterone at

TABLE 4. EFFECTS OF DEHYDROEPIANDROSTERONE IN INFANTILE FEMALE MICE

Status	Dose mg./day	Age at autopsy days	Preputial glands mg. ±S.E.	Phallus mg. ±S.E.
Ovarec (6)	—	28–32	3.84 ± .16	2.47 ± .35
Ovarec (8)	0.1	28–32	16.51 ± .92**	7.56 ± .32**
Intact (8)	—	27–28	4.20 ± .51	3.15 ± .31
Intact (6)	0.1	27–28	9.62 ± 1.52″	11.17 ± 1.08*
Male: C-4 (3)	—	25	—	4.19 ± .16
Male: intact (4)	—	28	—	34.9 ± 1.1

Explanation: Ovarec = ovariectomy, C = castration. Operations at four days of age, with implantation of subcutaneous DHA pellets. In the intact females, pellets were implanted on the day after birth. Numbers of mice in each group in ( ). Mean organ weights are followed by their standard errors. P values are indicated as. ** 0.001 or less; * 0.01 or less; ″ 0.02, relative to untreated controls for the respective groups. Organ weights in males for comparative orientation.

the same dose produced a marked response in both organs. However, at a dose of only 0.1 mg./day, testosterone produced a phallic growth 7 times that of the vesicles. Thus, with the smaller amounts of testosterone, or with the adrenal androgens in the doses used, phallic growth in immature animals is more responsive than is seminal vesicle growth, when the size increase is expressed as a percentage of the normal increment. The phallic enlargement produced by the adrenal androgens appeared to be grossly normal in the males.

In females DHA administration also produced a considerable enlargement of the phallus, as shown in Table 4, in both intact and ovariectomized animals. When treatment was begun on the day after birth, and continued for 4 weeks, the phallic weight attained about one third that of intact males of the same age. The distal parts were particularly enlarged, and the cavernosa appeared enlarged and highly vascular. However, the preputial skin covered only the ventral half of the phallus, producing a curious effect of hemisection. When treatment with DHA was initiated at 12 days

of age (mice of Table 2), there was no externally visible alteration in the genital tubercle, but at autopsy under magnification after deflection of the skin, the vascular cavernosa appeared considerably enlarged.

The distal half of the rodent phallus contains a sexually dimorphic bone which has been shown to exhibit a growth response to testosterone in hypophysectomized-castrated rats (30). Other parts of the skeleton in rats do not exhibit a growth response to testosterone in the absence of the pituitary (31). In gonadectomized mice of either sex there is a marked response of the phallic bone to the administration of DHA. The effects of castration and DHA treatment on the phallic bone in male mice are illustrated in Figure 9. Not only is there an increase in the length of the bone

FIG. 9. Phallic bones from litter mate male mice autopsied at 41 days of age. Left, from an intact male, showing adult form; center, from a male castrated at 4 days of age; right, from a male castrated at 4 days and implanted with a single pellet of DHA at 12 days. Steroid absorbed, 4 mg. in 29 days. Phallic weights were 46.6, 6.3 and 18.1 mg., respectively. The distal end (upper) retains the characteristic cartilagenous extension. ×20.

with DHA, but the changes in shape and thickness are striking. Qualitatively similar changes are produced by 11-hydroxy-Δ^4-dione.

DHA is probably present in human blood mainly as the sulphate (32). A test of DHA-sulphate showed it to be without appreciable activity in promoting phallic growth in mice castrated and implanted at 10 days of age and autopsied at 32 days. The DHA-sulphate was implanted at weekly intervals: two mice each absorbed 0.23 mg./day during the 22 day test period. Phallic/body weight ratios were: untreated control, 4.5; DHA-sulphate, 4.6; DHA 7.9. In this experiment the free DHA was absorbed at a rate of over 0.18 mg./day. The DHA-sulfate pellets were inclined to split, so that their greater rate of absorption may have been a result of available surface.

COMMENT

Early observations that the X zone in prepuberal male mice hypertrophies following castration, but normally is suppressed at the time of sexual maturity in males, indicated that the development of this zone was inversely related to the level of circulating testicular hormones (33). Histological evidence that the X zone is suppressed by testosterone administration has been presented in a number of studies, for example by Howard (34) and Chester Jones (16), (See also Howard and Migeon, 35).

In the present study it is shown that DHA and 11-hydroxy-Δ^4-dione produce a reduction in the weight of the adrenal in association with the histological changes, hence there must have been an actual suppression of certain elements of the adrenal by the androgens, not merely an alteration in the histological appearance. The finding that there is no weight reduction in the adrenal when adult males or females without X zone are treated with DHA indicates that this effect is relatively specific for the X zone.

Although methyl testosterone has been shown to reduce the adrenal growth response to ACTH in rats (36), and androgens may reduce ACTH secretion under some circumstances (36, 37), components of these effects did not become manifest under the conditions of the present experiments when intact adult males or females without X zone were treated with DHA. When DHA was given simultaneously with B, the suppression of the outer part of the cortex was more complete than with B alone. This might be a manifestation of either a reduced sensitivity to a low level of endogenous ACTH, a further reduction in, or a qualitative change of, pituitary secretion.

There is no direct contradiction between the present observations and those indicating that in hypophysectomized male rats androgens partially maintain the adrenals (38, 39). However, the situation is surprisingly complex, inasmuch as Winter *et al.* (39) reported that testosterone propionate or methylandrostenediol administered in conjunction with cortisone ace-

tate in rats prevented the adrenal atrophy which occurred when cortisone alone was given.

The complementary effects of DHA and corticosterone on the mouse adrenal reinforce other evidence of the independent control of the X zone and the zona fasciculata, recently reviewed (35). It appears, furthermore, that the X zone can be suppressed by DHA without apparently suppressing the maturation of the ovaries and uterus. On its face value, it might seem difficult to reconcile this observation with the conclusion that the X zone is maintained by a pituitary gonadotrophin (15). However, although DHA is presumably suppressing a pituitary gonadotrophin, it might also be entering into ovarian metabolism at some point after the site of action of the gonadotrophin. The conversion of androgens to estrogens by ovarian tissue has been clearly indicated (40, 41). It appears probable that ACTH acts on the adrenal to promote the conversion of cholesterol to pregnenolone (42). If a gonadotrophin acts on the ovary in an analogous way, one might expect that exogenous DHA would constitute an intermediary which could enter into ovarian metabolism and permit the ovary to elaborate estrogens at a considerable rate in the absence of the pituitary. This view finds supporting evidence in the work of Nelson and Merckel (10), who observed that DHA produced prolonged periods of estrus in hypophysectomized rats, with enlargement of the uteri, although DHA does not produce uterine enlargement in ovariectomized rats (6), and does not potentiate the action of estrogens (6, 9). If this interpretation is correct, DHA might be expected to suppress the pituitary gonadotrophin required for X zone maintenance without suppression of ovarian-uterine maturation. In addition, the possibility of a direct effect of DHA on the adrenal has not been excluded.

The dose of DHA which suppresses the X zone would be expected to exceed in androgenic potency any secretion of the adrenal when the X zone is present. In addition to suppressing the X zone, the doses of adrenal androgens studied produced considerable preputial and phallic growth. Since the same doses produced a relatively insignificant seminal vesicle enlargement, it may be concluded that previous failure to observe an influence of the adrenal on the size of the seminal vesicles in castrated mice (12) is consistent with the presence of some secretion of adrenal androgens. C_{19} steroids have been observed in mouse plasma (14).

The reproductive tract abnormalities which occur in congenital adrenal hyperplasia in females are characterized by enlargement of the phallus in the presence of relatively normal internal duct derivatives and ovaries (43). DHA and 11-hydroxy-Δ^4-androstenedione have been shown to be present in the human fetal adrenal, together with androstenedione (2). The latter is known to have a fairly high order of activity on seminal vesicles and phallus (44). Present observations indicate that the immature mouse

phallus is highly reactive to either DHA or 11-hydroxy-Δ^4-androstene dione. If the reactivity of the human embryonic phallus is similar to that the rodent, an oversecretion of any one of these three androgens in embryonic life might account for the phallic enlargement observed in human female infants with congenital adrenal hyperplasia. Alternatively, since DHA-sulphate was inactive, abnormal masculinization might be a consequence of a defect in the sulfation mechanism in some cases.

In normal human embryos, furthermore, it would seem possible that adrenal androgens contribute to the development of the phallus. By the time the embryo is 16–17 mm. in length the genital tubercle has elongated into a narrow conical organ which continues to enlarge for some time and constitutes a prominent structure of similar magnitude in embryos of either sex. Although sex differentiation is progressing, there is no appreciable sex difference in phallic size even when the embryo reaches 100 mm. in length (45). The adrenal cortex is differentiated with respect to its characteristic fetal zonation by 17–20 mm. (35) and the androgenic content of adrenal tissue was found to be at a maximum in embryos of 55 to 95 mm. (2).

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EFFECT OF GROWTH HORMONE AND ITS COMBINATION WITH ESTRADIOL-17β ON THE UTERUS OF HYPOPHYSECTOMIZED AND HYPOPHYSECTOMIZED-OVARIECTOMIZED RATS

RENZO GRATTAROLA[1] AND CHOH HAO LI

Hormone Research Laboratory, University of California, Berkeley, California

ABSTRACT

Growth hormone (somatotropin) induced an increase in uterine weight in both hypophysectomized and hypophysectomized-ovariectomized rats. This hormone exerted a direct effect on the uterus, as well as an indirect one through the ovary. No other pituitary gonadotropins (e.g., FSH, ICSH and lactogenic hormone) when injected singly increased significantly the uterine weight in hypophysectomized rats, but when growth hormone was injected with ICSH the uterine weight was greater than when growth hormone was injected alone. Growth hormone exerted a synergistic effect with estradiol-17β on the uterus of hypophysectomized and hypophysectomized-ovariectomized rats. These data furnish further evidence that somatotropin functions not only as a growth hormone but also as a biological synergist.

IN PREVIOUS studies, it was reported that bovine growth hormone maintained uterine weight in rats hypophysectomized at 26–28 days of age; moreover, the vaginas of these animals were open after hormonal treatment, despite the fact that the ovaries were atrophied (1). This paper represents an investigation on the effect of bovine growth hormone (somatotropin, STH) on the genital tract in order to ascertain whether this hormone acts directly on the uterus or through the ovary.

EXPERIMENTAL

Female rats of the Long-Evans strain were hypophysectomized at 21 days of age and, beginning fifteen days after operation, were given injections once daily for a period of 10 days. When ovariectomy or ovariectomy-adrenalectomy were performed, these operations were done immediately before the hypophysectomy and, as with the hypophysectomized groups, the injection period was begun 15 days later. Animals of the triply-operated group were maintained with injections of prednisolone (25 μg., Merck) every other day and with drinking water containing 1% NaCl and 5% glucose. Autopsy was performed 24 hours after the last injection. The uterus of each animal was weighed on a Roller-Smith torsion balance and, together with one ovary, was placed in Bouin's fixative. Later these organs were embedded in nitrocellulose, and 8μ sections were stained with hematoxylin and eosin. The other ovary was fixed in 10% formalin; subsequently

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it was infiltrated with 10% gelatin, and 10μ sections were made with a freezing microtome and stained with oil red in 60% isopropyl alcohol. Growth hormone was isolated from anterior lobes of bovine pituitaries by a simplified procedure (2) and was found to be free of contamination with any gonadotropic hormone when assayed at a dose level of 10 mg. in hypophysectomized rats (3). Lactogenic hormone (prolactin), follicle-stimulating hormone, and interstitial cell-stimulating hormone were prepared from sheep glands by previously described procedures (4, 5, 6). The pituitary hormone preparations were dissolved in distilled water and the estradiol-17β[2] was dissolved in sesame oil. All injections were given subcutaneously.

RESULTS

Removal of the ovaries from hypophysectomized rats resulted in a decrease in uterine weight (Table 1); however, no further decrease was observed when the adrenal glands were also removed. A daily dose of 0.05 mg. of growth hormone increased uterine weight to a significant extent in the hypophysectomized rats. Although this increment in uterine weight was less in the absence of the ovaries or ovaries·and adrenals, the uteri of the triply-operated rats were significantly heavier than those of the controls.

TABLE 1. EFFECT OF GROWTH HORMONE ON THE UTERINE WEIGHT OF HYPOPHYSECTOMIZED, HYPOPHYSECTOMIZED-OVARIECTOMIZED AND HYPOPHYSECTOMIZED-OVARIECTOMIZED-ADRENALECTOMIZED RATS

Animals	No. of rats	Daily* dose of GH	Body weight		Uterine weight
			at operation	at autopsy	
		mg.	gm.	gm.	mg.
Hypophysectomized	15	0	60.0	55.0	11.7 ± 0.5**
Hypophysectomized-ovariectomized	5	0	55.5	52.2	9.0 ± 0.4
Hypophysectomized-ovariectomized-adrenalectomized	6	0	53.3	51.3	9.6 ± 0.8
Hypophysectomized	10	0.05	54.0	66.6	17.2 ± 0.9
Hypophysectomized	12	0.50	54.7	69.2	21.5 ± 0.9
Hypophysectomized-ovariectomized	5	0.50	62.4	77.8	17.8 ± 1.1
Hypophysectomized-ovariectomized-adrenalectomized	7	0.50	50.1	68.4	14.2 ± 0.8

* Subcutaneous injections were begun 15 days after operations and continued for 10 days.
** Mean ± standard error.

Histologically, the uteri of the untreated rats were hypoplastic, and the ovaries contained only primordial follicles and a few degenerated follicles of medium size (Plate I, Fig. 1). In the treated animals, however, the endometrium was increased in thickness, as evidenced by slight folding and a higher epithelium. The connective tissue appeared less dense and more vascular than that of the controls. The cells of the germinal epithelium of the treated ovaries were cuboidal, and, in addition to primordial follicles, some follicles at the solid stage whose membrana granulosa contained healthy cuboidal cells were present. The interstitial cells were increased in

[2] Schering Corp. Bloomfield, New Jersey.

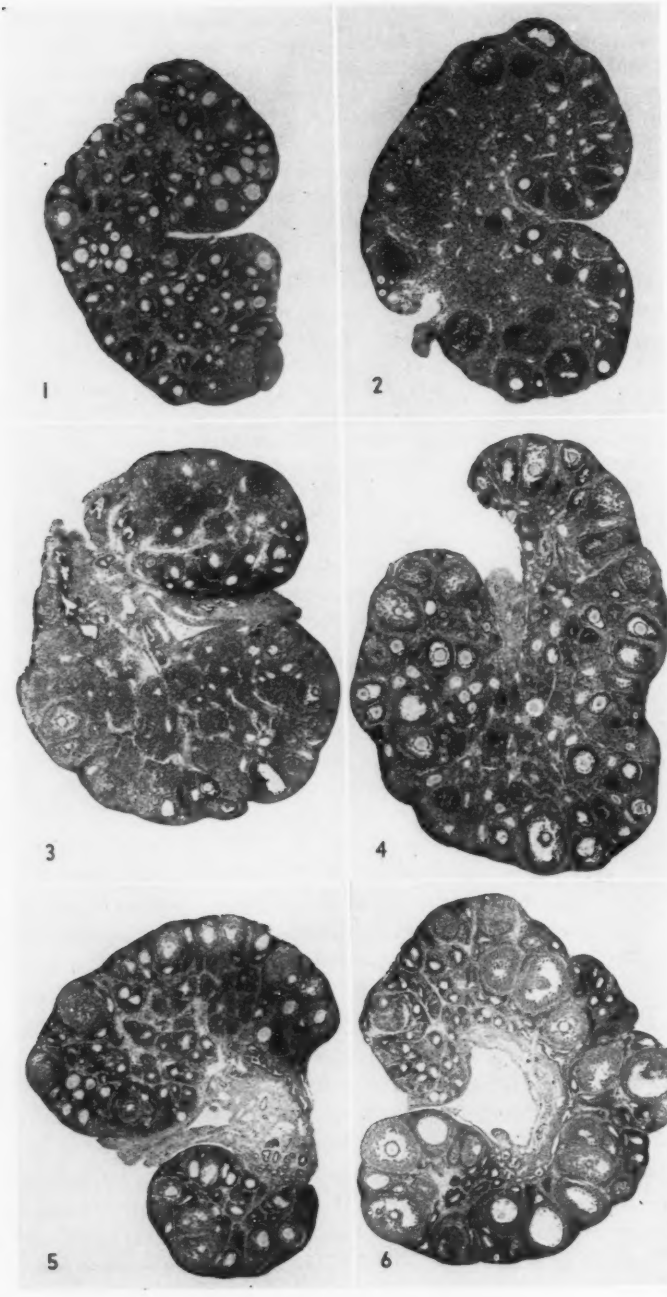
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TABLE 2. EFFECT OF GROWTH HORMONE AND OTHER PITUITARY HORMONES ON THE UTERINE WEIGHT OF HYPOPHYSECTOMIZED IMMATURE RATS

Treatment	Daily* dose	No. of rats	Body weights		Uterine weight	Ovarian weight
			Day of operation	At autopsy		
	mg.		gm.	gm.	mg.	mg.
Control	0	15	60.0	55.0	11.7±0.5**	5.0±0.4
Growth hormone	0.5	12	54.7	69.2	21.5±0.9	5.2±0.7
FSH	0.01	8	55.2	55.4	13.1±1.0	5.4±0.8
Prolactin	0.10	7	59.0	60.3	15.5±0.2	5.8±0.9
ICSH	0.10	5	56.4	55.6	10.8±0.6	6.3±0.4
ICSH + Growth hormone	0.10 + 0.5	11	53.0	79.4	27.0±2.5	8.0±0.1

* Hypophysectomized rats (operated on at 21 days of age) were injected subcutaneously, beginning on the 15th postoperative day, for 10 days.
** Mean ± standard error.

number and their cytoplasm was vacuolated (Plate I, Fig. 2) and rich in finely distributed lipid granules as shown by staining with oil red.

Growth hormone and, to a lesser degree, prolactin, elicited a significant increase in uterine weight (Table 2). An even more significant increase was observed in the animals treated with both ICSH and growth hormone. FSH, as would be expected, provoked marked follicular stimulation (Plate I, Fig. 6). In the prolactin-treated animals, most of the primordial follicles were degenerated, and a slight stimulation of the interstitial tissue had occurred (Plate I, Fig. 5). In the ovaries of the animals treated with ICSH, few follicles were present (Plate I, Fig. 3). The membrana granulosa was degenerated and the theca, which was highly vascular and contained enlarged vacuolated cells rich in finely distributed lipid granules, was increased in thickness. The interstitial cells were also slightly increased in size; the stimulation of the interstitial tissue elicited by ICSH was similar to that observed in the somatotropin-treated rats, but no increase of uterine weight was discernible. In the group treated with a combination of ICSH and growth hormone, the follicles were numerous with development

PLATE I

FIG. 1. Ovary of untreated hypophysectomized rat. (×35)

FIG. 2. Ovary of hypophysectomized rat treated with 500 μg. of growth hormone daily for 10 days. (×35)

FIG. 3. Ovary of hypophysectomized rat treated with 100 μg. of ICSH daily for 10 days. (×35)

FIG. 4. Ovary of hypophysectomized rat treated with a combination of 500 μg. of growth hormone and 100 μg. of ICSH daily for 10 days (×35)

FIG. 5. Ovary of hypophysectomized rat treated with 100 μg. of lactogenic hormone daily for 10 days (×35)

FIG. 6. Ovary of hypophysectomized rat treated with 100 μg. of FSH daily for 10 days (×35)

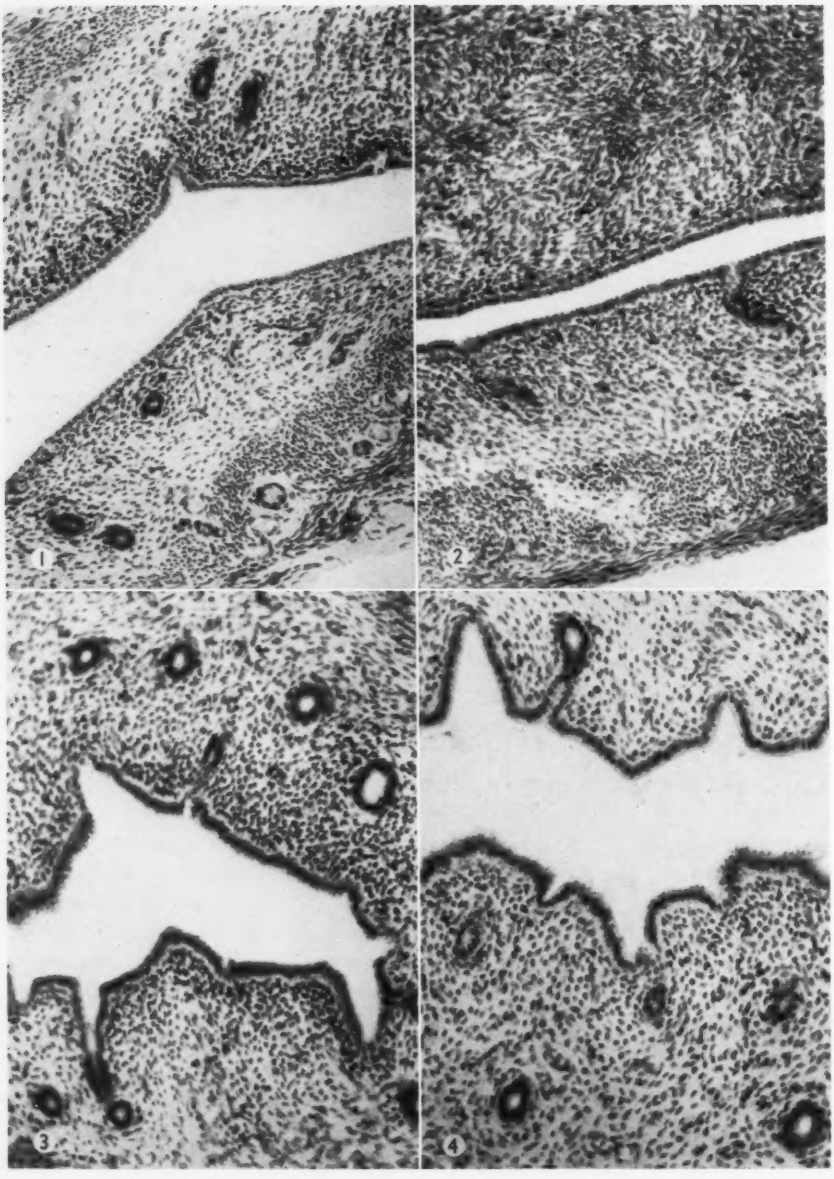

PLATE II

FIG. 1. Uterus of untreated hypophysectomized-ovariectomized rat (×127)

FIG. 2. Uterus of hypophysectomized-ovariectomized rat treated with 0.001 μg. of α-estradiol-17β daily for 10 days. (×127)

FIG. 3. Uterus of hypophysectomized-ovariectomized rat treated with 500 μg. of growth hormone daily for 10 days. (×127)

FIG. 4. Uterus of hypophysectomized-ovariectomized rat treated with a combination of 500 μg. of growth hormone and 0.001 μg. of α-estradiol-17β, given daily for 10 days. (×127)

to the antrum stage (Plate I, Fig. 4). The theca around the follicles was enlarged, with many vacuolated cells rich in lipid. The interstitial cells were more numerous and richer in fine lipid granules than when either ICSH or somatotropin alone was administered.

When the ovariectomized-hypophysectomized rats were injected with a daily dose of 0.001 μg. of estradiol-17β, no significant increase of the uterine weight above that of the control was observed, but when the same amount of estrogen was injected concurrently with somatotropin (500 μg.) the uterine weight was significantly higher than that obtained with somatotropin alone (Table 3). In the non-treated ovariectomized-hypophysectomized rats, the uterine wall was very thin. The surface epithelium (Plate II, Fig. 1) was low and the stroma was compact with a few pycnotic cells. Few glands were discernible. The uteri manifested a slightly higher epithelium

TABLE 3. EFFECT OF ESTRADIOL-17β, GROWTH HORMONE AND THEIR COMBINATION ON THE UTERINE WEIGHT OF HYPOPHYSECTOMIZED-OVARIECTOMIZED RATS

Treatment	Daily dose*	Body weights		Uterine weight
		Day of operation	At autopsy	
		gm.	gm.	mg.
Control		55.5	52.2	9.0 ± 0.4**
Growth hormone	0.500 mg.	62.4	77.8	17.8 ± 1.1
Estradiol-17β	0.001 mg.	57.2	57.0	10.8 ± 0.4
Estradiol-17β + Growth hormone	0.001 mg. + 0.500 mg.	56.4	72.8	25.2 ± 1.4

* Hypophysectomized-ovariectomized rats (operated on at 21 days of age) were injected daily, beginning on the 15th postoperative day, for 10 days; each group consisted of 5 animals.
** Mean ± standard error.

than that of the controls (Plate II, Fig. 2). Treatment with somatotropin (500 μg. daily dose) increased the thickness of the uterine wall. Slight endometrial folding was observed and the epithelium contained low columnar cells, although higher than that of the group treated with estradiol-17β (Plate II, Fig. 3). The glands were more numerous and their lumina more apparent. With the combined treatment (Plate II, Fig. 4) the uterine wall was thicker than with growth hormone alone. The stroma was edematous and rich in capillaries, and contained an abundance of large mesenchymal cells. The surface epithelium, with high columnar cells, protruded into the lumen. This phenomenon of a synergism between estradiol and growth hormone was also noted in hypophysectomized rats whose ovaries were intact. Growth hormone injected together with estradiol-17β always induced a greater increase in uterine weight than that obtained with the estrogen alone (Table 4). The difference between the uterine weights of the two groups was statistically significant. In all animals treated with the estrogen alone, the ovarian follicles had more layers of granulosa cells than did those

TABLE 4. EFFECT OF ESTRADIOL-17β, GROWTH HORMONE, AND THEIR COMBINATION ON THE UTERINE WEIGHT OF HYPOPHYSECTOMIZED RATS

Treatment	Daily* dose	No. of rats	Body weight		Uterine weight	Ovarian weight
			Day of operation	At autopsy		
	μg.		gm.	gm.	mg.	mg.
Control	0	15	60.0	55.0	11.7 ± 0.5**	5.0 ± 0.4
Estradiol-17β	0.0005	8	59.3	54.0	12.6 ± 0.6	6.1 ± 0.3
Growth hormone	500	12	54.7	69.2	21.5 ± 0.9	5.2 ± 0.7
Estradiol-17β + Growth hormone	0.0005 + 500	8	63.2	74.0	22.4 ± 1.1	7.2 ± 0.6
Estradiol-17β	0.001	6	56.5	56.0	15.2 ± 0.8	6.6 ± 0.1
Estradiol-17β + Growth hormone	0.001 + 500	6	58.3	73.6	23.3 ± 0.9	6.8 ± 1.5
Estradiol-17β	0.01	13	57.2	46.4	35.5 ± 0.5	4.2 ± 0.2
Estradiol-17β + Growth hormone	0.01 + 500	10	57.8	73.0	59.7 ± 2.2	7.5 ± 1.7
Estradiol-17β	0.1	8	57.6	55.0	157.2 ± 11.5	3.8 ± 0.4
Estradiol-17β + Growth hormone	0.1 + 500	10	60.5	65.5	191.1 ± 14.4	6.2 ± 0.1
Estradiol-17β	0.5	9	56.2	61.2	109.9 ± 9.1	4.9 ± 0.6
Estradiol-17β + Growth hormone	0.5 + 500	8	58.2	65.0	161.5 ± 14.5	5.0 ± 0.8

* Hypophysectomized rats (operated on at 21 days of age) were injected subcutaneously, beginning on the 15th postoperative day, for 10 days.
** Mean ± standard error.

of the controls, although no follicles at the antrum stage were observed. When somatotropin and estrogen were injected together, even more layers of granulosa cells were evident, in the follicles, which were still at the preantrum stage, and in addition, there was some enlargement of the interstitial cells. In animals treated with low dose levels (0.0005 μg. to 0.001 μg.) of estradiol alone, the surface of the endometrium was not folded and the cells of the surface epithelium were low and cuboidal. With the same amounts of estrogen plus growth hormone, the lumen, lined with columnar epithelium, was slightly folded. With higher doses of estradiol (0.1 and 0.5 μg.) high papillary projections were present, and the cells of the surface epithelium had swollen nuclei, with coarsely distributed chromatin and prominent nucleoli. When growth hormone was given concurrently with this high dose level of estrogen, the same high papillary projections were present but the

nuclei, although they still contained coarsely distributed chromatin, were not swollen and the nucleoli were not prominent.

DISCUSSION

The present study confirms earlier observations (7) that pituitary gonadotropins (FSH, ICSH and prolactin) alone are not capable of increasing uterine weight in hypophysectomized rats. That growth hormone when injected alone is able to stimulate uterine growth has been reported by Huggins *et al.* (8). We have demonstrated here that growth hormone acts directly on the uterus as well as by stimulating the interstitial cells in the ovary to produce an estrogen. That the interstitial cells are a source of estrogen has been shown by Engle (9), by Ingram and Mandl (10) and by Westman and others (11). In the present study, the cellular hyperplasia induced in the interstitial tissue was considered by us to be due to growth hormone and not to any gonadotropic contaminant. The amount of ICSH that will repair the deficient interstitial cells of rats after hypophysectomy will also enlarge the thecal cells and produce degeneration of the follicles (12). In this instance, growth hormone did not stimulate the thecal cells and during this hormonal treatment the follicles were free from any degenerative changes.

That growth hormone acts as a biological synergist (13) was clearly evident when it was injected concurrently with varying amounts of estradiol-17β in both hypophysectomized and hypophysectomized-ovariectomized rats. With this combined treatment, a greater increase in uterine weight was obtained than that observed when the estrogen was injected alone. Estrogens by themselves are able to promote an active growth of the uterus, but only to a certain dose level (14), beyond which there is a leveling off or "plateauing" of biological response. It has been observed (15), however, that when growth hormone was injected concurrently with that dose level of estrogen at which the plateau is reached, a further uterine growth was obtained. It is possible that growth hormone, as a protein anabolic agent (16) furnishes a sufficient accumulation of protein precursors in the uterus for the additional growth to proceed. Moreover, with respect to cellular structure, it has been shown (17) that estradiol interferes with the nucleoprotein metabolism of the endometrial cells so that an accumulation of ribonucleic acid occurs during treatment with estradiol, with an accompanying enlargement of the nucleoli. Histologically, we have observed that the epithelial cells and their nucleoli assumed a normal appearance when growth hormone was injected concurrently with the estrogen.

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ADRENOCORTICOSTEROIDS IN SALMON PLASMA (*ONCORHYNCHUS NERKA*)[1]

J. G. PHILLIPS,[2] W. N. HOLMES AND PHILIP K. BONDY

Bingham Oceanographic Laboratory, Yale University, New Haven, Connecticut, Department of Zoology, The University of British Columbia, Vancouver, Canada and Department of Internal Medicine, Yale University, New Haven, Connecticut

ABSTRACT

8,868 ml. of plasma were obtained from 364 male sockeye salmon (*Oncorhynchus nerka*), caught in the Stellako River, British Columbia, in October 1958. Extraction and analysis for adrenocorticosteroids revealed the presence of cortisol (5.2 μg./100 ml.), corticosterone (7.3 μg./100 ml.), cortisone (6.1 μg./100 ml.) and aldosterone (0.12 μg./100 ml.). In addition four unknown Δ^4-3-ketones possessing reducing side chains were also isolated and designated X_1 (0.63 μg./100 ml.), X_2 (0.1 μg./100 ml.) and W_2 and W_3 in approximately equal concentration (0.25 μg./100 ml.).

EARLIER studies have demonstrated that the main steroids, presumably of adrenocortical origin, found in fish blood or plasma are those which in the mammal are primarily concerned with carbohydrate metabolism (1–5). The control of water and salt-electrolyte metabolism in mammals is partly governed by the secretion of aldosterone (6–8). With the possible exception of the bird (3), aldosterone has not been identified in sub-eutherian vertebrates. Since the free aldosterone level of the blood of mammals is about 1μg./l. (6, 9), the failure to find this hormone in previous studies on fish may be due to the small volumes of plasma analyzed. It was decided, therefore, to collect a large volume of blood from a single species of fish living in an environment where the demand on the adrenal to secrete a possible salt retaining hormone would *a priori* be at its greatest. For this reason the sockeye salmon which had moved from sea water into fresh water to spawn were chosen for this investigation. In view of the reports of Hatey (10), Fontaine and Hatey (1) and the recent observations of Robertson (11), and Hane and Robertson (12) and Idler (13), it was decided also to separate and characterize as far as possible the cortisol-like hormones in the plasma.

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[2] Fellow of the Commonwealth Fund of New York. Supported by a grant to Dr. G. E. Pickford from the National Science Foundation (G 4001). Present address Department of Zoology, The University of Sheffield, Sheffield 10, England.

MATERIAL AND METHODS

Three hundred and sixty four male sockeye salmon (*Oncorhynchus nerka*) were taken from the Stellako River in October 1958, at a location about 150 miles West of Prince George in British Columbia. This particular race of sockeye salmon migrate to their spawning ground from the Pacific Ocean via the Fraser and Nechako Rivers. The total migratory run from the estuary of the Fraser to their spawning ground on the Stellako is covered in approximately 28 days at an average of 36 kilometers a day (14). The fish were caught in a seine net in groups of about thirty in number and kept in a retaining net in the river until used. All the fish were either in the act of spawning or had spawned.

Blood collection. Each fish was removed from the net, wiped dry, and the urogenital openings clamped with a curved hemostat. This precaution was taken to prevent contamination of the blood sample. A collar of paper towelling wrapped around the tail prevented water and slime from dripping off the body into the collecting vessel. Blood was obtained by severing the tail and holding the fish vertically over a heparinized vessel, approximately 50–60 ml. of blood being obtained from each fish. No clotting took place. The blood was centrifuged within 15 minutes of collection and the separated plasma was removed into polyethylene bottles surrounded by dry ice. In this way 8,868 ml. of plasma were collected and pooled. The frozen plasma was transported to the University of British Columbia where it was extracted and the dried extract was shipped by air to Yale University in a refrigerated vessel sealed under nitrogen.

Extraction. Before extraction 44.49 ml. of a standard ethanolic solution of cortisol-4-C^{14} containing 2330 counts per minute per ml. was added to the plasma by placing appropriate amounts of the solution into the extracting vessels and evaporating the ethanol off under a stream of nitrogen. The plasma was then added to the vessel and

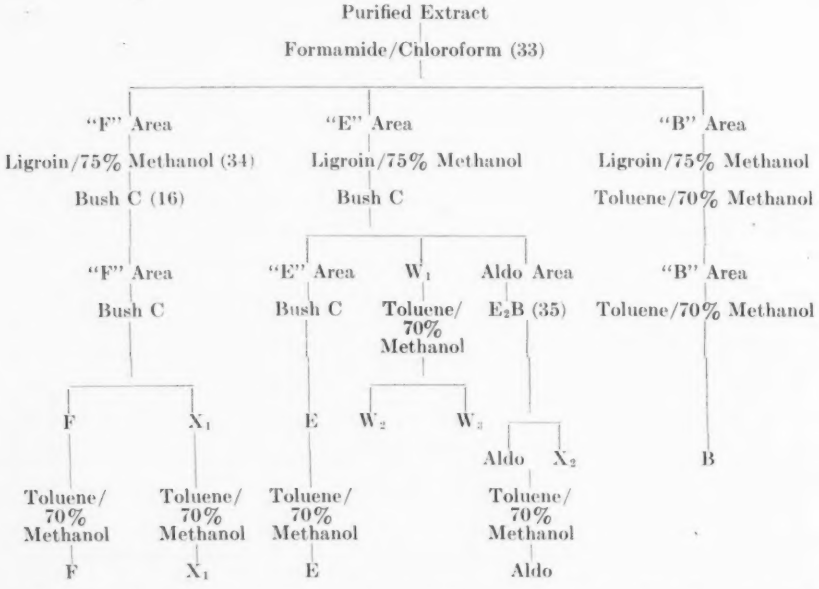

FIG. 1. Scheme of chromatographic separation of steroids in chloroform extract of salmon (*Oncorhynchus nerka*) F, cortisol: E, cortisone: B, corticosterone: Aldo, aldosterone. X_1, X_2, W_2, and W_3, unknowns.

allowed to stand for a few minutes with occasional stirring, so that the cortisol-4-C^{14} was evenly distributed throughout the plasma. To every liter of plasma, 25 ml. of 1 N NaOH was added and the mixture promptly extracted by shaking four times with a liter of redistilled chloroform (18). The chloroform extracts were combined and evaporated *in vacuo* to near dryness at 37° C. All traces of chloroform were removed with nitrogen. The residue at this point weighed 1.2364 gm. and to it was added a tracer amount of aldosterone-16-H^3 (0.0016 μg.). The residue was purified by partition between redistilled n-hexane and 50% ethanol as described by Carstensen (15). The aqueous ethanol was reduced to 1/10 vol. in a flash evaporator and the remaining aqueous solution transferred to a separatory funnel and extracted four times with $\frac{1}{3}$ vol. redistilled chloroform. The combined chloroform extracts were reduced to dryness in a stream of nitrogen and the resulting residue was sufficiently small for direct application to paper.

Chromatography. The purified extract was analyzed by paper chromatography which followed the plan outlined in Figure 1.

Characterization. The following procedures were employed to characterize the hormones extracted from the plasma.

1. Comparison of the chromatographic mobilities of the plasma steroids with those of authentic standard reference steroids run in parallel in several chromatographic systems (see Fig. 1).
2. For cortisol and aldosterone, mixed chromatography with cortisol-4-C^{14} and aldosterone-16-H^3 respectively.
3. Reflex photography in ultra violet light (16).
4. Simultaneous detection of a reducing side chain and the α,β-unsaturated-3-ketone grouping of cortical steroids by spraying the chromatograms with a freshly prepared solution of 5 mg. 2,3,5 triphenyltetrazolium chloride in 100 ml. 2N NaOH (17).

Estimation of steroid levels. Accurate estimation of the steroids was carried out by the methods of Bondy *et al.* (18) using cortisol-4-C^{14} as a tracer and making a correction based on isotope dilution for losses during the processing of the plasma.

RESULTS

Type of secretion. Eight α,β-unsaturated-3-ketones were separated from the plasma extract. All of them reduced triphenyltetrazolium chloride and four were identical in their chromatographic behavior with cortisol, cortisone, aldosterone and corticosterone. The structure of four compounds designated X_1, X_2, W_2 and W_3 is unknown at present. X_1 was eluted off the chlorofororm/formamide system along with the cortisol area but it was separated from and was more polar than cortisol in the Bush C system; on over-running in the toluene/70% methanol system this substance retained its polar position between cortisol and the origin of the chromatogram. W_1 ran with cortisone in the formamide/chloroform system but was more polar than cortisol and aldosterone in Bush C system. On re-chromatography in the toluene/70% methanol system W_1 split into two spots (W_2 and W_3) containing approximately equal amounts of steroidal material, both being more polar than cortisol. X_2 was eluted along with cortisone from the formamide/chloroform system and together with aldosterone it was separated from cortisone in Bush C system. The aldosterone area from Bush C when chromatographed in the E_2B system separated into two distinct absorbing spots; one ran with authentic aldosterone run in parallel

and the second (X_2) was more polar than corticosterone and less polar than cortisone. Further evidence for the authenticity of the cortisol and aldosterone isolated from the plasma was provided by mixed chromatography with cortisol-4-C^{14} and aldosterone-16-H^3. Of the added cortisol-4-C^{14},42.8% appeared in the cortisol spot in the final chromatogram and 50% of the added tritiated aldosterone was found in the aldosterone spot after the E_2B system. Radioactivity was insignificant in the remaining parts of the chromatograms.

Levels of secretion. Estimation of levels of steroid hormones isolated from the plasma are included in Table 1.

Concentrations of cortisol, cortisone, and corticosterone are roughly equal whilst aldosterone appears in trace amounts. The level of cortisol is

TABLE 1

	Total steroid isolated (μg.)	Corrected# recovery	Less tracer steroid	μg./100 ml. plasma
Cortisol	224	523.4	460.5	5.2
Cortisone	233.1	544.6*	544.6	6.1
Corticosterone	276.0	644.9†	644.9	7.3
Aldosterone	4.4	10.3*	10.3‡	0.12
X_1	24.0	56.1*	56.1	0.63
X_2	3.65	8.5*	8.5	0.1
W_1	19.2	44.9*	44.9	0.51

Accurate recovery data is only available for cortisol (42.8%) since this was the only labeled steroid added before extraction.

* It is presumed that steroids of similar polarity to cortisol would incur losses during chemical manipulations of a similar order to cortisol. The values have been accordingly adjusted but must be considered as approximate only.

† Losses for corticosterone are probably greater than for cortisol (see text and reference 19). This figure is therefore minimal.

‡ Added labeled aldosterone (0.0016 μg.) too small for subtraction. Aldosterone-16-H^3 added to purified extract (before chromatographing) and showed a 50% recovery.

an accurate estimation whilst that of cortisone, corticosterone and aldosterone is calculated on the assumption that losses during chemical manipulations and chromatography would inflict approximately the same losses on these substances as for cortisol. Further the level recorded for corticosterone is almost certainly an underestimate since on partition between hexane and aqueous ethanol, corticosterone is probably partially lost into the hexane phase (19). Estimation of the unknown compounds shows them to be in much lower concentrations than the familiar steroids isolated, excluding aldosterone.

DISCUSSION

The characterization and significance of the presence of the unknown compounds awaits further investigation. Cortisol and/or corticosterone have been found in many species of fish (3, 4) but cortisone has not been previously detected. Cortisone has been found as a component of adrenal secretion in mammals (17, 20), in the snake and capon (3) and in *in vitro*

preparations of *Fundulus* interrenals (21). It was interesting to find appreciable amounts of cortisone in salmon blood since this hormone, supposedly a secretion from the human adrenal (22) is not detectable in peripheral blood samples in this species. However, Bush and Ferguson (17) have shown that cortisone can appear as an artifact, being an oxidative product of cortisol. This possibility can be ruled out in the present investigation since insignificant amounts of radioactivity could be detected in the isolated cortisone, indicating that no conversion of the added cortisol-4-C^{14} to cortisone-4-C^{14} had taken place during chemical manipulations.

The occurrence of a mineralocorticoid and glucocorticoids in fish plasma strongly suggests that, as in mammals, the biological activity of the adrenal secretion in fish can be accounted for by these two types of secretion. The biological function of these hormones is less clear in fish than it is in mammals. Even in the latter class, the available evidence suggests that a strict division into glucocorticoids and mineralocorticoids is one of convenience and that an overlapping of function occurs in their effects on carbohydrate and salt-electrolyte metabolism. In this connection Nace (23, 24) has shown that intramuscular injections of DCA, 11-dehydro-17-hydroxycorticosterone-21-acetate and 17-hydroxycorticosterone-21-acetate will raise the blood sugar levels of the toadfish (*Opsanus tau*). Further, it has been demonstrated (5) that a glucocorticoid (cortisol) and a mineralocorticoid (DCA) have similar effects on salt movements in the rainbow trout (*Salmo gairdneri kamloops*). It was shown that the rainbow trout, presented with a salt load followed by treatment either with cortisol or DCA reacted to these hormones by retention of Na at the kidney level, inhibition of Na re-uptake from the environment and promotion of secretion of this ion through the gills. The conditions of these experiments were special ones in that the trout were previously loaded with sodium chloride and the increased excretion of sodium when the blood contains large amounts of this ion may reflect particular circumstances not shown in their normal environment. We can conclude, however, that adrenocortical hormones of both types can act on the gills as well as the kidney. Contrary to Chester Jones *et al.* (5), Sexton (25) has shown that in the *in vitro* preparation of goldfish gills, cortisone acetate had no effect on sodium uptake or loss either in the normal or salt loaded animals. DCA on the other hand, caused a depression of sodium uptake as it did in the trout but had no effect on sodium loss from the gill. Clearly, the whole problem needs re-investigation now that aldosterone is known to be a normal component of adrenal secretion in the sockeye salmon and its production *in vitro* has been demonstrated from interrenal preparations obtained from *Fundulus heteroclitus* (21).

Pickford and Atz (26) have reviewed the evidence which suggests that a pituitary-adrenal axis operates in fish. In particular Rinfret and Hane (27) have shown that the salmon pituitary contains ACTH. Further, Robertson

and Wexler (28) have demonstrated a marked adrenocortical hyperplasia attributed to pituitary overstimulation in three species of spawning Pacific salmon and they suggest that the death of the salmon is caused by oversecretion of catabolic hormones of adrenal origin. In support of this idea, Robertson (11) was able to show that implants of cortisol in cholesterol pellets caused the death of rainbow trout.

Robertson (11) and Hane and Robertson (12) have recently found that the plasma 17-hydroxycorticosteroids in the king salmon (*Oncorhynchus tschawytscha*) are low in immature sea-run fish (11.8 μg./100 ml). On the spawning migration there is a 4 to 5-fold increase (males: 45.1, females: 53.4μg./100 ml.). In the female at the time of spawning the level rises to 78.7μg./100 ml. whilst there is a decline to 31.3μg./100 ml. in the male. The combined 17-hydroxycorticosteroid levels are much lower in male sockeye salmon taken at the spawning grounds (cortisol + cortisone = 11.3μg./100 ml). The data of Idler *et al.* (13) indicate that, as in the king salmon, much higher levels are present in fish taken on the spawning migration. Just prior to arrival at the Adams river spawning grounds, they found that the plasma of sockeye salmon contained 17 μg./100 ml. of cortisol and 37 μg./100 ml. of cortisone. Unfortunately the sex was not stated and the high values may result, in part, from the presumed admixture of female plasma with that of males. It would be expected that the act of migration would impose heavy demands on the physiology of the fish since a migration of 1000 kilometers is accomplished in a relatively short time without feeding (14). The results of Leloup-Hatey are consistent with the view that increased muscular activity involves the adrenal gland. She was able to show in the carp that forced swimming for progressively longer periods of time causes an initial increase in cortisol levels and after one hour of forced swimming corticosterone appears in addition (4). A decline in cortisol levels which occurs after two hours of forced swimming might reflect an inability of the adrenal to continue its enhanced secretory rate. Further, Fontaine and Hatey (1) attributed the elevated 17-hydroxycorticosteroid level in the smolt stage of *Salmo salar* to the need for muscular activity concomitant with its seaward migration and the low levels of the parr stage to reflect an absence of this need. Active swimming (comparable to the experimental swimming of Leloup-Hatey's fish) over a long period of time is required of the salmon to reach the spawning grounds. The physiological mechanisms involved in both carp and salmon may be similar but an unknown factor operating in the salmon and not in the carp, enables the salmon to maintain prolonged excercise.

These results foreshadow many interesting investigations. The earlier work on the mammalian adrenal (29, 30), in which a division of secretory function between the zona glomerulosa producing mainly mineralocorticoids and the zona fasciculata producing glucocorticoids has received more direct experimental proof (31). The results of hypophysectomy indicate

that the glomerulosa enjoys an autonomy not possessed by the remaining zones. Hypophysectomy in lower vertebrates (26, 32) results in atrophic changes which are not uniform throughout the gland. Some cells retain a normal histological appearance. Since the adrenal of fish is not zoned histologically it will be interesting to determine whether there is a functional division of cellular components in an apparently homogeneous interrenal as there is in the zoned adrenal cortex of the Eutherian mammals.

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EFFECT OF HYPOTHALAMIC LESIONS ON PROGESTERONE-INDUCED OVULATION IN THE HEN

C. L. RALPH[1] AND R. M. FRAPS

Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland

ABSTRACT

Previous work has implicated a ventro-median region in the preoptic hypothalamus of the chicken in the control of normal ovulation. To determine whether or not the hypothalamus is involved also in progesterone-induced ovulation, electrolytic lesions were placed by means of a stereotaxic instrument at various sites within the hypothalamus of birds previously injected with 1 mg. of progesterone. Only those lesions placed within a ventro-median portion of the preoptic hypothalamus (the same region involved in normal ovulation), or along fiber tracts passing caudad from this region regularly prevented ovulations that ordinarily would have been induced by progesterone. Furthermore, only lesions placed in the effective region within about 2 hours after injection of progesterone prevented ovulation. Lesions placed at later times had no influence on progesterone-induced ovulation (but either abolished or delayed subsequent ovulations.).

The ventro-median hypothalamic region could be either the actual site of progesterone "excitation" or merely a link in a neural complex mediating the effects of progesterone. Destruction of this region apparently precludes the release of gonadotrophic hormone which usually follows when progesterone is administered at appropriate times.

PROGESTERONE regularly induces premature ovulation of the first follicle of the hen's sequence following the subcutaneous injection of 1 mg. per hen some 14 hours before expected normal ovulation (1, 2). There is strong but indirect evidence that this steroid induces ovulation by acting upon a neural mechanism to cause liberation of ovulation-inducing hormone (OIH).

Pharmacological studies have contributed most to this concept. Zarrow and Bastian (3) reported that while progesterone alone caused 90% of their hens to ovulate, only 20% ovulated following treatment with atropine and progesterone. Fraps (4, 5) found that phenobarbital sodium prevented most expected ovulations when injected prior to a highly effective level of progesterone. The number of hens ovulating following progesterone administration is greatly reduced if Dibenamine, which is believed to be an adrenergic blocking agent, is injected simultaneously (6). This observation was extended by van Tienhoven (7) who described what appears to be a

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[1] Present address: Department of Biological Sciences, University of Pittsburgh, Pittsburgh 13, Pennsylvania.

cholinergic component as well, based upon suppression of ovulation with atropine. Furthermore, near subovulatory dosages of progesterone act synergistically with the barbituates Dial and Nembutal to produce premature ovulation (8).

Rothchild (9) found that progesterone solutions applied directly to the surface of the pituitary were no more effective in inducing ovulation than when placed upon the sphenoid bone covering the gland or even on an exposed area of the thigh. His conclusion was that progesterone acts not directly upon the pituitary to release ovulating hormone but through some systemic mechanism.

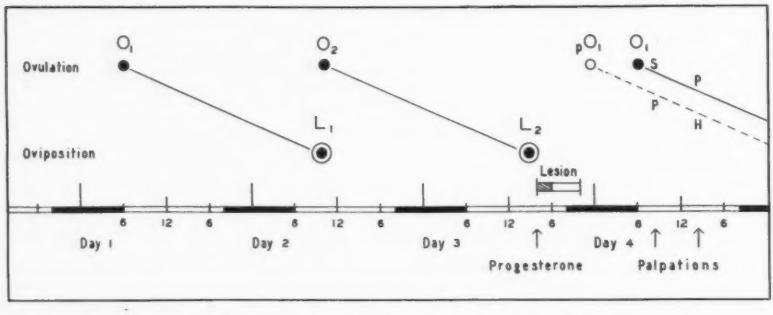

FIG. 1. The design of the experiments illustrated with a typical two-egg sequence. O_1 and O_2—first and second ovulations in a sequence. L_1 and L_2—lay of first and second eggs. Progesterone was administered at 4:00 p.m. Lesions were made during the following 7 hours; cross-hatching in bar indicates time interval during which lesions were effective in preventing ovulation. $_pO_1$—a premature O_1 not prevented by lesions. The oviduct was palpated for the presence of an egg at 8:30 a.m. and 2:30 p.m. the following day. S, P, H—soft, plump, and hard, indicating the state of the egg in the oviduct as determined by palpations.

In another report (10) it was suggested that normal ovulation depends upon the integrity of certain parts of the hypothalamus and that destruction of these by electrolytic lesions may immediately halt the process of ovulation. Experiments were undertaken to determine whether the same hypothalamic regions are parts of the presumed neural mechanism actuated by progesterone. The results reported here indicate that this is so. A preliminary account has been published (11).

MATERIALS AND METHODS

A direct approach to the problem of neural participation in the ovulation process has been made possible by the use of a stereotaxic instrument fitted with a head-holder adapted for the chicken. Details of the stereotaxic and lesioning procedures employed are given elsewhere (10).

The experimental animals were White Leghorn hens 9 months to 2 years of age. They were maintained in laying batteries and given food and water *ad libitum*. Artificial

illumination, daylight not excluded, was provided from 6 a.m. to 8 p.m. Time of lay was recorded hourly from 8 a.m. to 4 p.m. Birds selected for experiment were laying sequences of two to four eggs each, mostly two-egg sequences. Each experiment was concerned with the first ovulation of a sequence only.

Progesterone dissolved in corn oil was injected subcutaneously, 1 mg. per hen, between 4:00 and 4:15 p.m. into three to eight birds on the day of lay of the terminal egg of a sequence. The interval between injection of the steroid and ovulation is from 7 to 8 hours (5). Premature ovulation of the first follicle of the next sequence, if allowed to occur, would then take place between 11:00 and 12:00 p.m. Lesions were placed at selected intervals within 7 hours after the injection of progesterone. The occurrence or failure of ovulation was determined by palpating the oviduct through the cloaca the following day (Fig. 1).

Electrolytic lesions, made with a direct-current coagulator, were placed either singly on the midline or 0.5 to 2 mm. bilaterally. Combinations of currents ranging from 1 to 3 milliamperes and durations of 5 to 25 seconds were employed in the various experiments, creating lesions 0.5 to 1.5 mm. in diameter. When sacrificed, all birds were perfused through the heart, their brains were removed, fixed in formalin, and sectioned while frozen and stained with thionin. Some series were also stained with sudan black B to demonstrate fibers.

The luteinizing hormone preparation used in one experiment was Armour's Pituitary Gonadotrophin (LH) dissolved in distilled water.

RESULTS

Twenty-five experiments like that illustrated by Figure 1 have been carried out, the position and size of the lesions having been varied in several ways. Two major items of information have been gained: (1) the possible sites of progesterone "excitation" and (2) the duration of its action in the hypothalamus. Lesions that prevented ovulation were limited to either the anterior part of the ventro-median hypothalamus, in what appears to be a ventral portion of the nucleus praeopticus paraventricularis (12), or to fiber tracts, originating in the ventral preoptic region and passing through the supraoptic region (Fig. 2). At no other sites in the region probed was it found possible regularly to prevent ovulation. Lesions on the midline or 0.5 to 1 mm. bilaterally were all equally effective in preventing induced ovulations. These are the same hypothalamic regions reported previously (10) to participate in normal ovulation. When placed 2 mm. bilaterally the lesions proved ineffective.

The properly positioned lesions must be made within 2 to $2\frac{1}{2}$ hours after injection of progesterone to prevent subsequent ovulation, as is demonstrated by the experiments summarized in Table 1. The duration of progesterone action in the hypothalamus thus appears to be about 2 hours. Lesions placed in the effective regions after this period have no influence on the subsequent progesterone-induced ovulation (but will, of course, affect later ovulations).

A few ovulations did occur when lesions were made in the effective region and before the end of the critical 2-hour period (cf. Fig. 2). Failure to block ovulation may be attributed, in at least some of these cases, to in-

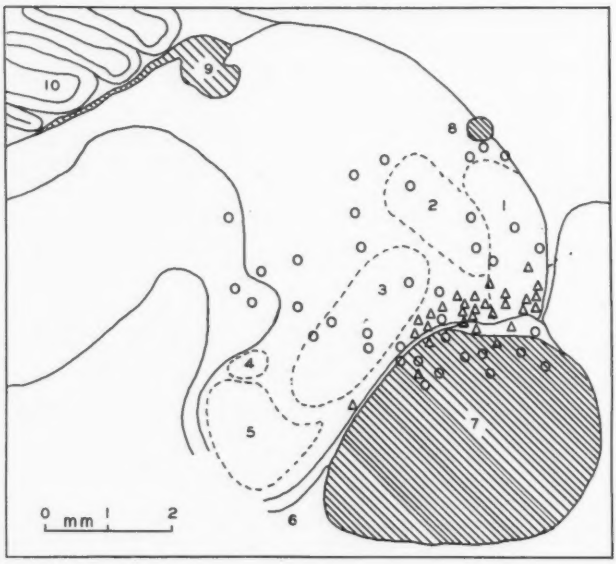

FIG. 2. Mid-sagittal plane of chicken diencephalon on which certain hypothalamic nuclei and the sites of all lesions made within two hours following administration of progesterone are projected. Symbols indicate the approximate center of each lesion: O—ovulation; Δ—failure of ovulation. 1 and 2—principal and accessory parts of preoptic paraventricular nucleus, 3—lateral hypothalamic nucleus, 4—mamillary nucleus, 5—tuberal nucleus, 6—median eminence, 7—optic chiasma, 8—anterior commissure, 9—posterior commissure, 10—cerebellum. Fiber tracts not shown. Limits of nuclei are somewhat arbitrary.

accurate bilateral placement of the lesions, as was determined by examining the sectioned brains. In others the lesions may have been too small to destroy enough of the participating neurones. Well defined small lesions that appeared to be placed in comparable positions often varied in effectiveness in preventing ovulation. However, when the lesions were 1 to 1.5 mm. in diameter and properly placed, blockage of the next expected ovulation was accomplished.

Evidence that prevention of progesterone-induced ovulation by lesions is not caused by impairment of ovulatory responsiveness of the follicle (at least not immediately) was demonstrated by the effectiveness of luteinizing hormone injected intravenously at the time of subcutaneous injection of progesterone. Bilateral lesions were placed in the effective preoptic region of five birds within 2 hours after the 4:00 p.m. injections. All five of the birds ovulated prematurely. It would be expected that most or all birds bearing lesions, if not injected with LH, would have failed to respond to progesterone. Response to the direct action of exogenous gonadotrophin shows that the follicle is still ovulable. Failure of progesterone to induce

TABLE 1. MINIMAL DURATION OF STIMULATION OF THE HYPOTHALAMUS BY PROGESTERONE[1] AS DEMONSTRATED BY LESIONS PLACED IN THE VENTRAL PREOPTIC HYPOTHALAMUS[2]

Time of injection	Time of operation	Interval	Response[3]
4:07 P.M.	5:10 P.M.	1′30″	N
4:09	6:09	2′00″	O
4:10	7:06	2′56″	O
4:11	8:11	4′00″	O
4:12	9:12	5′00″	O
4:13	10:43	6′30″	O
4:00 P.M.	4:30 P.M.	0′30″	N
4:01	5:01	1′00″	N
4:02	5:32	1′30″	N
4:03	6:33	2′00″	O
4:04	7:34	3′30″	O
4:05	8:35	4′30″	O
4:01 P.M.	5:31 P.M.	1′30″	N
4:02	5:52	1′50″	N
4:03	6:13	2′10″	N
4:04	6:34	2′30″	O
4:05	6:55	2′50″	O

[1] Injected subcutaneously, 1 mg./hen.
[2] Lesions produced by 2.5 ma. for 25 seconds in the first experiment, 2.5 ma. for 20 seconds in the second, and 2.0 ma. for 10 seconds in the last.
[3] N—no ovulation; O—ovulation.

ovulation after placement of lesions is thus apparently the result of partial or complete lack of OIH (or LH) release.

DISCUSSION

The preoptic hypothalamus is undoubtedly concerned with gonadotrophin liberation following progesterone administration. Only lesions placed in a small region of the paraventricular nucleus or in fibers connecting that region with the hypophyseal structures prevented progesterone-induced ovulation, suggesting that certain neurones of the paraventricular group are the site of progesterone "excitation." However, the possibility remains that these neurones and their fibers are merely links in a structural complex. Although anatomical evidence lends some support to the former view (13), the latter possibility cannot be excluded.

The timing relationships suggested by these experiments agree very closely with the findings of Rothchild and Fraps (2) that removal of the pituitary within 2 hours after the injection of progesterone prevented ovulation. In addition, van Tienhoven (7) has calculated that a cholinergic component in progesterone-induced ovulation is functional for no more than $2\frac{1}{2}$ hours following progesterone injection.

It appears, therefore, that release of ovulation-inducing hormone from the pituitary occurs in close association with activation of the hypothalamus, since both phases of the process are completed about 2 hours after injection of progesterone. van Tienhoven (7) suggested earlier that stimulation of neural processes and release of LH from the hen's anterior pitui-

tary gland proceed concurrently following the administration of progesterone.

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PROGESTERONE AND 4-PREGNEN-20α-OL-3-ONE IN THE TISSUES OF PREGNANT RATS[1]

WALTER G. WIEST

Department of Biological Chemistry, University of Utah College of Medicine, Salt Lake City, Utah

ABSTRACT

Concentrations of two progestational hormones, progesterone and 4-pregnen-20α-ol-3-one, were estimated in tissues of pregnant rats in the last third of pregnancy. Measurable quantities of both compounds were obtained from ovaries (progesterone, 7.9 μg. per gm.; 4-pregnen-20α-ol-3-one, 10.1 μg. per gm.), blood (progesterone, 0.07 μg. per gm.; 4-pregnen-20α-ol-3-one, 0.03 μg. per gm.), and fat (progesterone, 0.12 μg. per gm.; 4-pregnen-20α-ol-3-one, 0.08 μg. per gm.). Fetuses, placentae, amniotic fluid and uteri on analyses gave negative findings. The conclusion was reached that ovaries are the major source of progestational hormones in the rat during late pregnancy.

THE chemical isolation of progestational material from the rat has not been reported previously; however, recent developments of sufficiently sensitive techniques (1–6) have made possible the chemical assay of three related steroids, progesterone, 4-pregnen-20α-ol-3-one and 4-pregnen-20β-ol-3-one, in tissues from human beings and from several animal species. The present report deals with the isolation of progesterone and 4-pregnen-20α-ol-3-one from the tissues of pregnant rats.

EXPERIMENTAL

Fifty-four pregnant female rats, selected on the 13th to 19th days after the appearance of a vaginal plug, were exsanguinated into heparinized syringes via the abdominal aorta while under ether anesthesia, and the blood was immediately centrifuged. An average of 11.5 ml. of blood was obtained from each animal yielding 6.0 ml. of plasma. Embryos and placentae were dissected promptly from each uterus. Umbilical cords and fetal membranes were combined with amniotic fluid collected as quantitatively as possible following rupture of the membranes. The embryos were washed with normal saline, and the washings were added to the amniotic fluid fraction. The residual uteri, ovaries and aliquots of peritoneal fat were also removed for analysis. Combined fluids and tissues from 5 to 20 rats were analyzed in batches, and homogenates of the tissues were prepared in distilled water with the aid of a Lourdes Multimixer (20 gm. tissue per 100 ml. water). Lipid extracts were then prepared from plasma, amniotic fluid, and tissue homogenates according to the method of Zander and Simmer (1), which on purification

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provided a steroid-containing solution essentially free of neutral fat that could be chromatographed on paper strips 2 cm. wide in the heptane-formamide system (7) with two hours overrunning.

Scanning the dried chromatograms at 240 mμ in a Cary recording spectrophotometer equipped with a special attachment for moving the strips past the light beam (8) provided a means of estimating quantitatively the steroids present in the extracts. Chromatograms obtained from extracts of large quantities of tissue (100–200 gm.) gave high background absorption which necessitated rechromatography and rescanning. When recordings of the chromatograms indicated normal background levels, the areas of any absorption peaks corresponding in mobility to the three known gestagens were determined by planimetry. Measurement of absorption peaks for standard amounts of authentic progesterone chromatographed in the same way provided data for a standard curve (Fig. 1). Steroid equivalents were then estimated from the standard curve. The

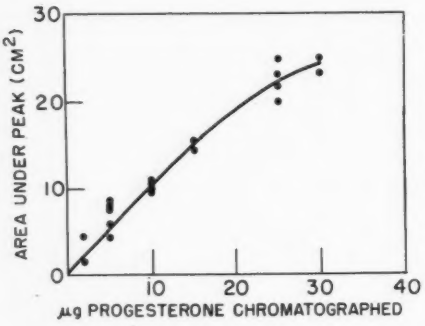

FIG. 1. Standard curve for estimating amounts of gestagens in extracts of blood and tissues. Indicated amounts of authentic progesterone were chromatographed on paper strips 2 cm. wide in the heptane-formamide system (7) with two hours overrunning. Dried paper strips were then scanned in a Cary recording spectrophotometer at 240 mμ. Areas of the absorption peaks were measured by planimetry.

extinction coefficient of 4-pregnen-20α-ol-3-one was assumed to be the same as that for progesterone.

Characterization of the progestational substances obtained from tissue extracts was based on their chromatographic mobilities, their ultra-violet absorption maxima and sulfuric acid spectra, and upon the formation of chemical derivatives.

RESULTS

Ovaries.—Four separate analyses were made of pooled ovaries. Two substances (5 to 8 μg.) having chromatographic mobilities identical with progesterone and 4-pregnen-20α-ol-3-one could be estimated accurately on the amount of material obtained from 10 ovaries. The concentrations of these two substances are given in Table 1.

Plasma.—Results from these analyses were inconclusive. Although plasma volumes ranging from 31 to 111 ml. were extracted, in the majority of cases no material having ultra-violet absorption at 240 mμ was detectable; however, in some of the studies, compounds having mobilities comparable with progesterone and 4-pregnen-20α-ol-3-one were found but not in sufficient quantities to be accurately measurable. It would appear that the concentrations of these steroids in plasma from pregnant rats are less than 0.1 μg./gm. of plasma (Table 1).

Fat tissue.—From 52 gm. of peritoneal fat significant quantities of progesterone (6.5 μg.) and 4-pregnen-20α-ol-3-one (4.2 μg.) were isolated. The concentrations in this tissue are also given in Table 1.

Fetuses, placentae, amniotic fluid and uteri.—Chromatograms of the extracts of these tissues failed to demonstrate the presence of ultra-violet absorbing steroidal material in high enough concentrations for significant measurement. The quantities of tissue used for each analysis are shown in Table 1.

Analyses of all of the above tissues failed to demonstrate the presence of 4-pregnen-20β-ol-3-one. All of the chromatograms prepared during this study showed some pigmented material which remained on the origin, and

TABLE 1. LEVELS OF PROGESTERNOE AND 4-PREGNEN-20α-OL-3-ONE IN PREGNANT RAT TISSUES (13TH TO 19TH DAY OF PREGNANCY)

Tissue	Weight	Progesterone	4-Pregnen-20α-ol-3-one
	gm.	μg./gm.	μg./gm.
Ovary	0.734	10.3	8.2
Ovary	0.648	9.3	12.4
Ovary	1.327	5.3	10.6
Ovary	1.383	6.5	9.3
Plasma	31.0	0.07	0.03
Fat (peritoneal)	52.27	0.12	0.08
Placentae	71.20	not detectable	not detectable
Amniotic fluid	96.40	not detectable	not detectable
Uterus	56.00	not detectable	not detectable
Fetuses	119.20	not detectable	not detectable

which could have obscured any steroids more polar than 4-pregnen-20α-ol-3-one. Since the study was designed to measure only the three recognized natural gestagens, this more polar fraction was not examined further.

Characterization of steroids.—Identification of peak A, Figure 2, as 4-pregnen-20α-ol-3-one was based on the following properties: 1) Its chromatographic mobility was identical with that of the authentic steroid. 2) It possessed an ultra-violet absorption maximum at 240 mμ. 3) It gave a yellow-brown color on paper chromatograms treated with I_2-KI which characteristically changed gradually after about 2 minutes to royal blue beginning at the periphery and extending finally throughout the entire spot. This blue color was stable for 15–20 minutes, fading slowly. 4) Acetylation to a compound having the properties of authentic 4-pregnen-20α-ol-3-one acetate. 5) Oxidation with CrO_3 to progesterone. Similar criteria were used for the identification of 4-pregnen-20α-ol-3-one obtained from eviscerated rats previously injected with progesterone (9).

Identification of peak B as progesterone was based on its following properties: 1) Its chromatographic mobility was identical with that of the authentic compound. 2) It possessed an ultra-violet absorption maximum

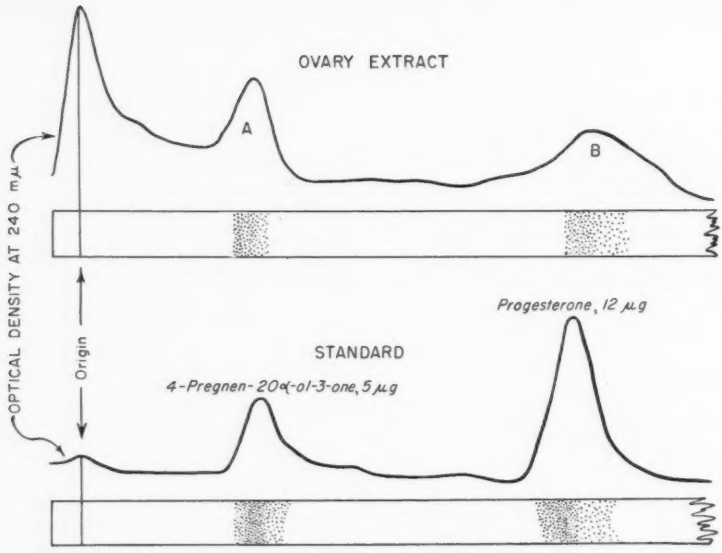

FIG. 2. Facsimiles of chromatograms containing standard amounts of authentic progesterone and pregnen-20α-ol-3-one (bottom) and the extract from 10 pregnant rat ovaries (top). Shaded portions represent areas of absorption visible when viewed by ultra-violet light. The curves correspond to the tracings obtained from the Cary recording spectrophotometer.

at 240 mμ. 3) It gave a sulfuric acid chromogen having an ultra-violet maximum at 290 mμ.

DISCUSSION

The pioneer work of Zander and coworkers (1, 10–14) has resulted in the isolation and characterization of progesterone, 4-pregnen-20α-ol-3-one and 4-pregnen-20β-ol-3-one from human blood and tissues. Other methods for the quantitative determination of progesterone in human plasma have been described by Butt *et al.* (6), Short (2), Sommerville (4), and Oertel *et al.* (5), and in human placental tissue by several workers including Pearlman and Cerceo (15), Salhanick *et al.* (16, 17), Diczfalusy (18), Zander and von Münstermann (14), and Haskins (19).

The progesterone content of the body fluids and tissues of several animals has been studied (3, 20–24). Circulating levels of the hormone have been determined during pregnancy in the cow by Short (21) and throughout the estrous cycle and pregnancy in the ewe by Edgar and Ronaldson (23).

In addition to the findings of Zander *et al.* already cited, the presence of 4-pregnen-20α-ol-3-one has been observed in human placentae (25, 26), in peripheral blood of the ewe (20), and in adrenal vein blood of the calf (27),

and the 20β epimer has been found in mare placenta (20, 26) and in bovine ovaries (22).

The metabolic relationship between progesterone and 4-pregnen-20α-ol-3-one has been demonstrated in the eviscerated rat (9). It is clear that reduction of the 20-keto group does not require participation of the liver (28). Hayano *et al.* (29) isolated 4-pregnen-20β-ol-3-one after incubation of cattle corpora lutea with progesterone.

Demonstration of the biological activity of 4-pregnen-20α-ol-3-one and 4-pregnen-20β-ol-3-one in the Clauberg test and the Hooker-Forbes test (20, 31) led Zander *et al.* to conclude that these two compounds along with progesterone should be regarded as naturally occurring gestagens.

It is generally agreed that in the rat oophorectomy during pregnancy results in fetal death and abortion (32). However, it would appear that under restricted experimental conditions the ovaries are not essential for the maintenance of pregnancy (32–36), although the presence of the progestational hormone is. The placenta has been considered as a source of gestagens (34); however, the weight of evidence supports the conclusions that the ovaries remain the major site of gestagen production in the rat throughout pregnancy, and that the placenta while capable of gestagen synthesis is a relatively minor source compared with the ovaries. The chemical studies reported here are in full agreement with these conclusions.

Acknowledgments

The author expresses to Dr. Leo T. Samuels his appreciation for the valuable suggestions received during the conduct of this work.

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DEUTERON IRRADIATION OF THE MONKEY PITUITARY[1]

M. E. SIMPSON, G. VAN WAGENEN, D. C. VAN DYKE, A. A. KONEFF AND C. A. TOBIAS

Institute of Experimental Biology and Department of Anatomy, Donner Laboratory of Biophysics and Medical Physics, University of California, Berkeley, California; Department of Obstetrics and Gynecology, Yale Medical School, New Haven, Connecticut

ABSTRACT

Observations on the effects of pituitary irradiation have been extended to the monkey. An effort has been made to determine whether irradiation exerts a selective effect on the functions of different cell types in the pituitary, and to determine the dosage necessary for complete destruction of the pituitary. The pituitaries of a group of young monkeys of known age, 4 females and one male, were irradiated with a beam of high energy deuterons. The pituitaries of three monkeys received 9000 rep, two received half this dose, or 4500 rep. All were irradiated when immature, between 8 mo. and 1 yr. 3 mo. of age. They were observed for post-irradiation periods of 2 yr. 4 mo. to 4 yr. 4 mo. The radiation was localized in the pituitary by passage of the beam through an aperture of the size and the shape of the pituitary. A high concentration of the radiation in the pituitary with only limited irradiation of other tissues in the path of the beam was achieved by rotation of the animals during irradiation. Only slight damage was noted in adjacent tissues, including nerves, blood vessels, and brain. No hemorrhages occurred from the internal carotid or the circle of Willis.

1. It was possible to irradiate the monkey pituitary with single doses of 4500 and 9000 rep deuterons, without serious injury to nerves or to those parts of the brain lying in the path of the beam. A remarkable localization of the lesion to the pituitary was achieved.
2. The vascular trunks so near the pituitary were not severely damaged; no rupture of these vessels occurred and they were not occluded by thrombi. No gross hemorrhages were found in the pituitary, though minute hemorrhages occurred. Some thickening of vascular walls was present in the small vessels of the meninges of the pituitary and of the base of the brain, particularly in the region of the infundibulum.
3. Deuteron irradiation reduced the size of the pituitary. Only the higher dose caused disorganization of lobular architecture with profound destruction of parenchyma and replacement by fibrous tissue. At least a few normal appearing chromophils could be found in the anterior lobes of all pituitaries.

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4. The types of damage were similar in all lobes. No specific cell type of the anterior pituitary appeared to be particularly sensitive to radiation.
5. A differential effect on the pituitary target organs was noted. After 9000 rep there was complete suppression of development of the ovary, and marked interference with growth, but little evidence of damage to the adrenals and thyroid. At the lower dose, 4500 rep, the ovary functioned for a time and then failed; body growth slowed but did not cease.
6. A definite time-lag occurred between pituitary irradiation and full expression of the damaging effects. Two females (4500 rep) matured sexually, menstruated for 5 and 10 months, and became amenorrheic 2 and $2\frac{1}{2}$ years after irradiation. In 3 of 5 irradiated monkeys several months elapsed (5 to 15) before growth rate slackened.
7. Evidence of late recovery from irradiation was noted in one male and one female, in which after complete statis for 5 and 10 months respectively, body growth was resumed at a slow rate.
8. No adenomatous transformations in pituitary cells, such as those observed in the rat, were noted in irradiated monkey pituitaries.

FOLLOWING the demonstration that the pituitary gland of rats can be selectively destroyed by a well localized beam of accelerated high energy nuclei from the 184″ cyclotron (1, 2) this method of altering pituitary function has been applied to the primates, monkey (*Macaca mulatta*) and man (3). This report deals with the long term observations made on a small group of young monkeys after deuteron irradiation of the pituitary gland.

METHOD

The procedure for the alignment of the pituitary in the path of the deuteron beam, the size of the aperture, the roentgenographic check of alignment and the method of measurement of radiation dose and its spread are essentially the same as those explained in detail in previous publications. The monkeys were anesthetized with Nembutal and placed in a supine position in a specially designed holder which provided firm fixation of the head by means of plugs in the external auditory canals and a pin between the maxillary incisors (Fig. 1). The head could be rotated during irradiation through a total arc of 90° ($\pm 45°$), the beam passing through the sella turcica as the center of rotation. All monkeys were rotated in a single plane through this angle except two which were rotated in nine different planes ($\theta = 0$, $\pm 7°$, $\pm 15°$, $\pm 22°$, $\pm 30°$ as in chart 3, reference 3). (In irradiating the pituitaries of rats it was not necessary to rotate the head since the morphological relationships are such that the beam could be passed through the pituitary without passing through the central nervous system.) The beam was aligned by superimposing the beam image on lateral roentgenograms, the deuteron beam and the X-ray having been aligned previously for parallax (Figs. 2 and 3). Alignment during rotation was assured by proper centering of the bony landmarks on an anterior-posterior roentgenogram taken with 90° rotation of the head (Fig. 4). The deuteron beam was collimated to conform to dimensions of the pituitary by being passed through an aperture slightly larger than the pituitary; one animal received radiation through a round aperture 6 mm. in diameter, the remainder were irradiated through an aperture shaped to conform more closely to the dimensions of the pituitary, 4×6 mm. (Table 1). The actual size of the pituitary did not exceed 4 mm. (superior-inferior) by 5 mm. (anterior-posterior).

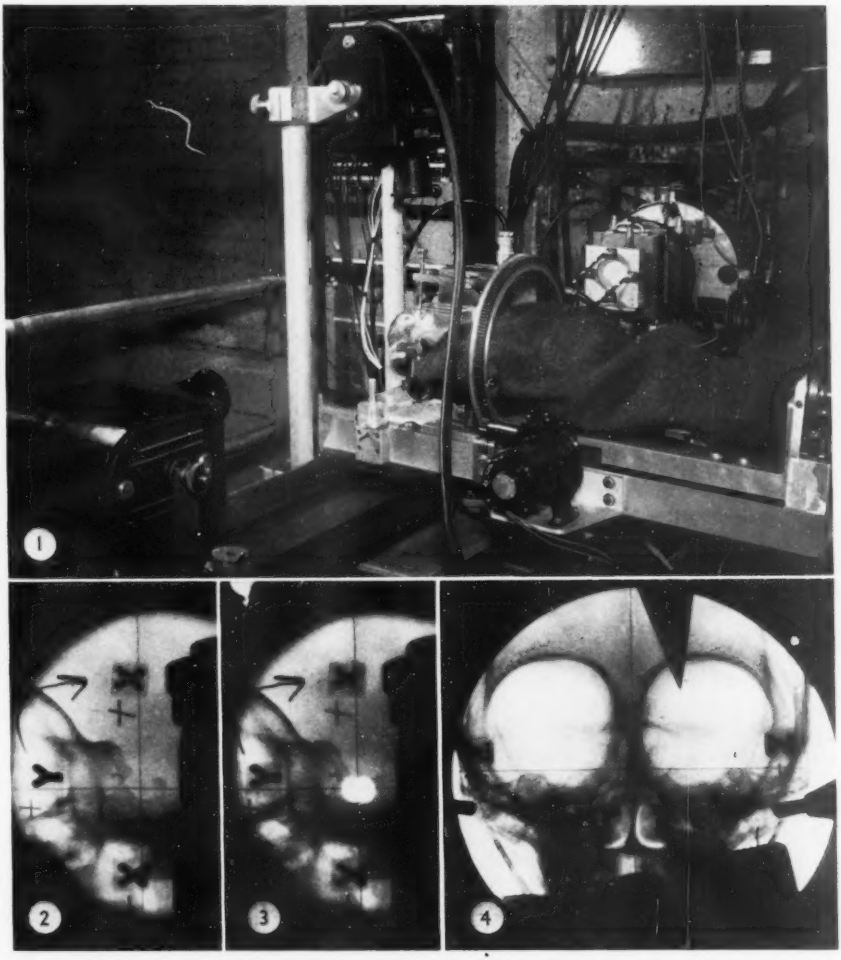


FIG. 1. An anesthetized monkey during irradiation, with the head firmly fixed on the rotating platform. One X-ray unit is aligned to the beam port and the second is perpendicular to the axis of the beam. The gear and driving mechanism for lateral rotation are shown.

FIGS. 2 to 4. Roentgenograms of the skull of Mm 856 taken in preparation for irradiation. (X and Y notations were used to assist in alignment of the cross hairs to the sella turcica.)

2. Lateral view showing cross hairs intersecting at the center of the sella turcica.
3. A second exposure in the same position as fig. 2, with the image of the beam superimposed.
4. Anterior-posterior view taken to demonstrate the alignment of the vertical cross hair in the mid-sagittal plane to insure proper positioning during rotation.

TABLE 1. CONDITIONS IN DEUTERON IRRADIATION OF THE MONKEY PITUITARY

Sex	Monkey Number	Dose rep	Age yr.-mo.-da.		Interval Between Radiation and Autopsy yr.-mo.-da.	Conditions of Irradiation
			At Radiation	At Autopsy		
Females	856	9000	1-1-24 (7/19/'55)	3-5-19 (11/14/'57)	2-3-26	aperture oblong; 90° rotation in multiple (9) planes
	839	9000	0-8-9 (1/19/'54)	4-6-5 (11/15/'57)	3-9-27	aperture round; 90° rotation in 1 plane
	862	4500	0-11-0 (7/19/'55)	3-2-26 (11/15/'57)	2-3-27	aperture oblong; 90° rotation in (9) planes
	903	4500	ca 1-3-0 (7/14/'53)	ca 5-0-0 (11/15/'57)	4-4-2	aperture oblong; 90° rotation in 1 plane
	887	Controls	—	3-6-22	—	—
	695			5-2-0		
	946			ca 4-5-0		
Males	837	9000	0-9-4 (1/19/'54)	2-2-3 (6/18/'55)	1-4-29	aperture oblong; 90° rotation in 1 plane
	838	Control	—	2-2-4 (6/25/'55)	—	—

The dose was measured by a parallel plate ionization chamber similar to that used previously in this series of studies (1–3). The actual dose was checked in a phantom of approximately the same dimensions as those of the head of the monkey. Photographic films which had been properly calibrated were inserted in this phantom and the isodose distribution curves were measured. The isodose curves proved to be approximately ellipsoids which conformed closely to the shape of the pituitary gland. The doses measured at the ionization chamber were 5000 and 10,000 rep,[2] and were delivered at the rate of approximately 500 rep per minute. The actual measured isodose curves are given in a graph plotted as percent of the dose against millimeter distance from the center of the pituitary gland (Fig. 5). The dose falls off rapidly in the direction of the hypothalamus, optic chiasma and brain stem, but less rapidly, due to the particular method of rotation, laterally in the region of the temporal lobe and the cranial nerves. Many factors enter into the calculation of the absolute dose delivered to the pituitary; for example, the increase in ionization along the Bragg ionization curve, the actual divergence of the beam, contamination of the deuteron beam by other particles, and its attenuation as it passes through tissue. These factors accounted for an 11% decrease from the calculated dose. The actual doses at the geometrical center of the pituitary, calculated on the basis of the isodose curves, and modified by these considerations, were 4500 and 9000 rep respectively. The doses are accurate to approximately 10%.

Two females and one male monkey were irradiated with 9000 rep and two females with 4500 rep. The male was the only animal in which any injury to the cranial nerves was noted in spite of the close proximity of these structures to the pituitary; a transient ptosis of the eyelid and a dilated pupil on the right side occurred in this animal. Table 1

[2] In conformity with the usage in previous papers (1 and 2) dealing with irradiation of the pituitary of the rat, doses are reported here in rep (roentgen equivalent physical). The new international 1 rad or 1.07 rep = 100 erg absorbed per gm. tissue.

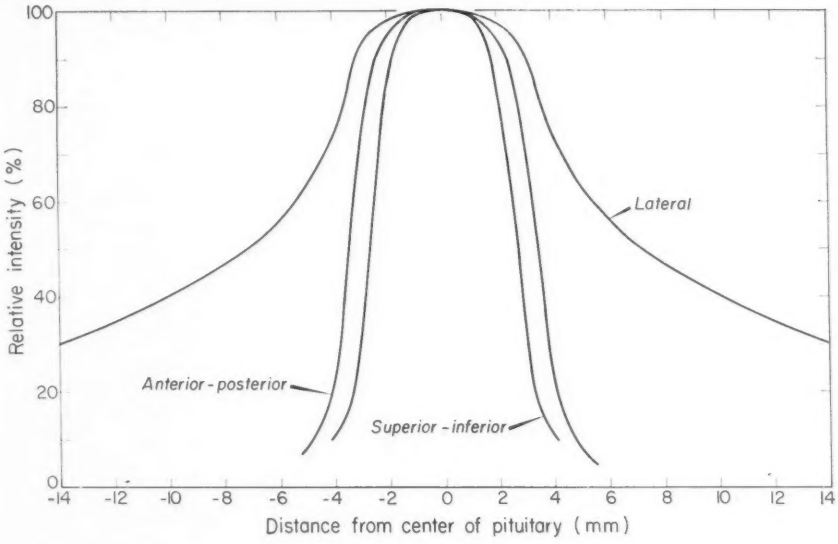


FIG. 5. Isodose curves showing beam intensity distribution; diameter of pituitary 4 mm. (superior-inferior) $\times$ 5 mm. (anterior-posterior). Aperture size 4 $\times$ 6 mm.

(The greater lateral spread results from the fact that the beam was introduced laterally with rotation.)

summarizes the conditions of the experiment, the sex of the monkeys, the age at irradiation and at autopsy, the dosage and the variability in aperture and rotation used during irradiation.

The monkeys (with one exception) were of known age and lineage. The following observations were made during the post-irradiation period, extending in some instances for four years.[3] The animals were weighed at regular intervals. Skeletal growth and differentiation were followed by routine measurements of sitting height, and periodic skeletal roentgenograms. The animals were observed carefully for evidences of sexual maturation (vaginal desquamation, development and changes in the sex skin and menstruation). Blood counts, total circulating red cell volume, and thyroid iodine uptake were determined.

The monkeys were fed three times a day. One meal was cooked, a carbohydrate base of rice, macaroni, or potatoes being used as a carrier for incorporation of adequate proteins, milk, peas, and beans, and of salts and liver extract. The cooked meal was supplemented by fresh fruits and vegetables, as well as fresh milk, and vitamins administered individually. The body weight was difficult to maintain, especially in the animals which received the higher dose of radiation. Their appetite was not good, so that in addition to the regular diet the irradiated monkeys received more frequent feedings, and when ill were given subcutaneous saline clysis. Although the temperature in the colony did not fall below 65° F the irradiated monkeys were housed in the nursery due to their sensitiveness to temperature change. They were frail and susceptible to infections and needed

[3] Joseph Negri was responsible for the daily record of teeth, menstrual cycle, etc., and for the meticulous care required to maintain these delicate animals in good health over periods of 2 to 4 years.

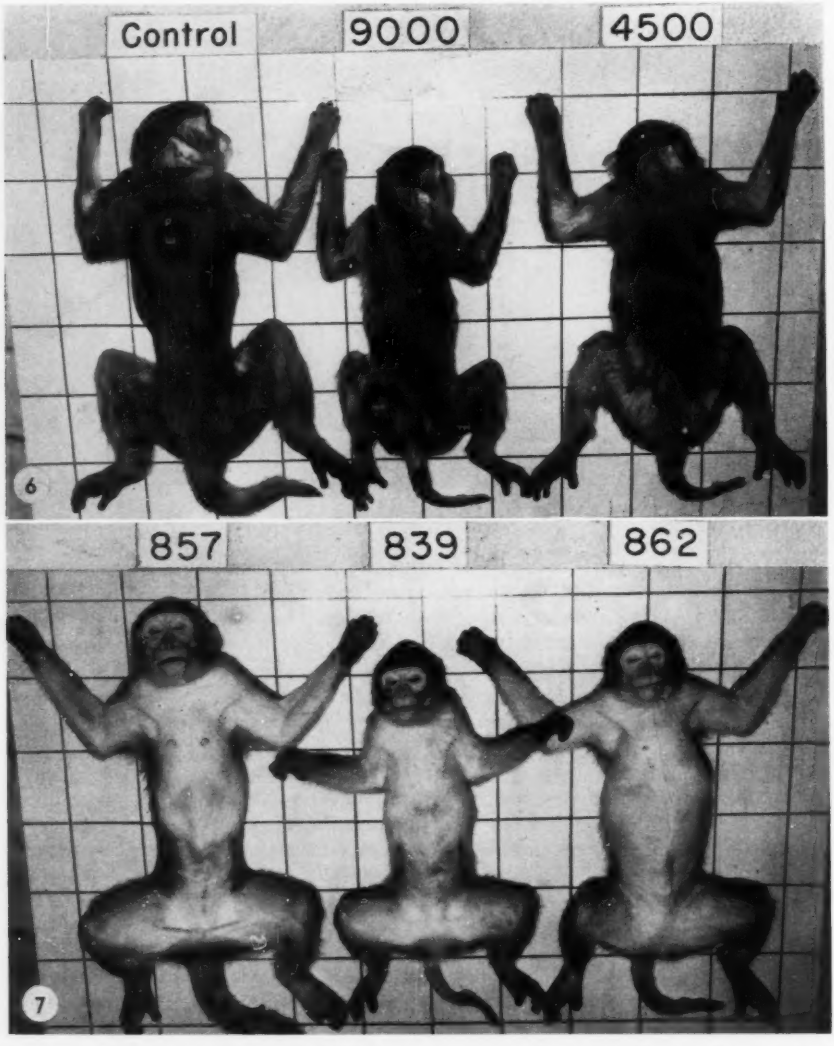


FIG. 6. Photographs of dorsal view. Control and 2 experimental females. The numbers of the individual monkeys are indicated on the photographs, as are the dosages of radiation (rep) to the pituitary.

FIG. 7. Ventral view: animals arranged in the same order.

watchful care. Though they were surprisingly active, they had to be protected from harassment by other animals. Those which were irradiated with 9000 rep could not compete for food with those which had received 4500 rep, and in turn these animals could not compete with normal animals. The physical strength and muscle tone was diminished in the same ratio. The irradiated monkeys were housed for companionship

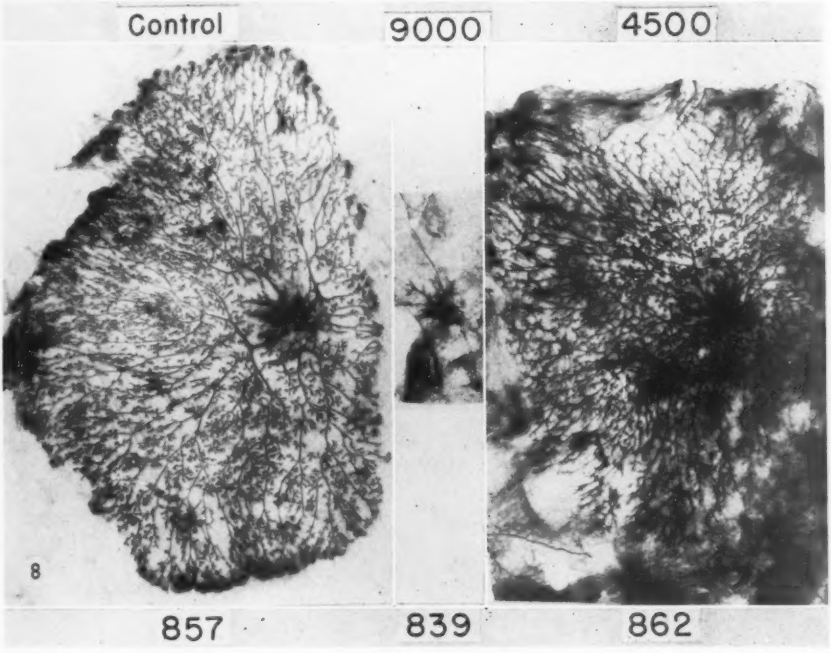


FIG. 8. Mammary spreads from the same control and two irradiated female monkeys as shown in Figs. 6 and 7.

next to younger and younger animals as time passed, until finally 5 year-old monkeys were housed in cages adjacent to those of 6 month infants.

RESULTS

Body contours. The irradiated monkeys, though of adult age at the termination of the experiment, were small and slender and of juvenile contour (Figs. 6 and 7). Compared with controls the trunk was short, the shoulders and hips were narrow and the animals were less slender through the lower trunk region than adult multiparous animals of the same chronological age. The head was smaller, and the vertex-mandibular measurements were less than in controls; the teeth were crowded, indicating impaired jaw development.

Pelage. The hair of irradiated monkeys was soft, fine, short and less glossy than in controls. The cape hair was shorter and the hair of the lower sides extended less far toward the ventral midline (Fig. 7).

Body growth. The rate of body weight increase was retarded after irradiation—more after 9000 rep, less after 4500 rep. The growth of male and female monkeys irradiated between 8 mo. and 9 mo. of age with 9000 rep reached a plateau within 3 to 4 mo.; growth stasis continued for 5 mo. in

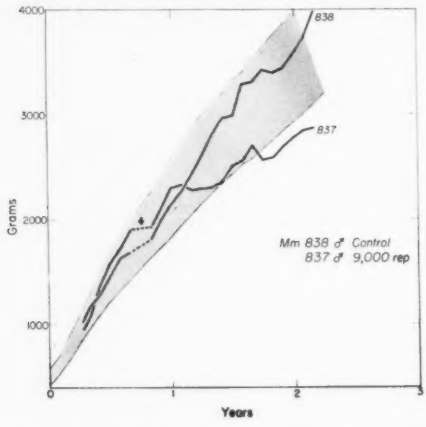


FIG. 9. Increase in body weight in male monkeys; Mm 837 irradiated with 9000 rep, and control Mm 838. Shaded area: control values (one S.D. from mean).

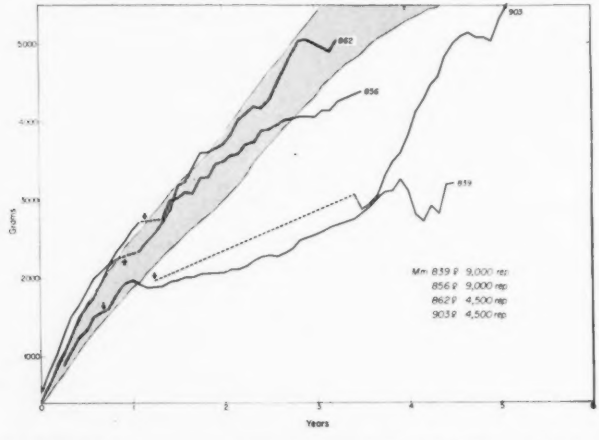


FIG. 10. Body weight increase in females; Mm 856 and 839 irradiated with 9000 rep and Mm 862 and 903, 4500 rep. Shaded area: control values (one S.D. from mean).

Mm 837 and 10 mo. in Mm 839 after which growth was resumed at a slow rate. Growth in the remainder of the monkeys (those irradiated with 9000 or with 4500 rep between 11 mo. and 1 yr. 3 mo. of age) did not lag behind that of the contemporary controls until 10 mo. after irradiation (Figs. 9, 10). The skeletal growth measured by the increase in sitting height is shown in figures 11, 12. The shaded area represents one standard deviation from the colony average (4). The discrepancy between predicted and actual sitting height was greater in monkeys which received the higher doses. The growth rate of Mm 856, which received 9000 rep, paralleled that of the

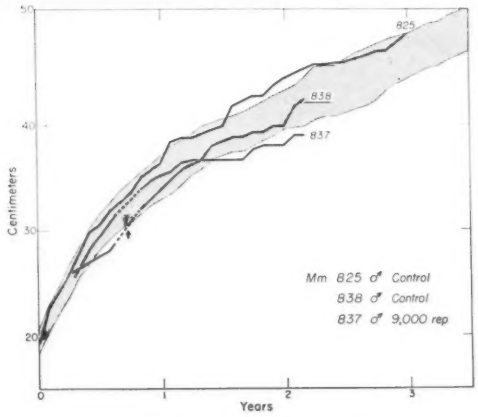


FIG. 11. Increase in sitting height in irradiated male Mm 837, and contemporary controls, Mm 838 and Mm 825. Shaded area: control values (one S.D. from mean).

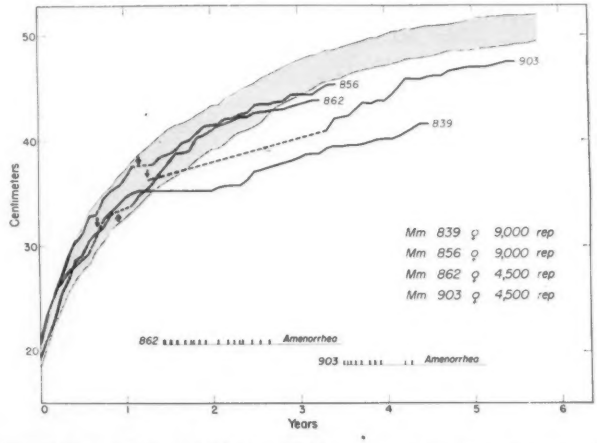
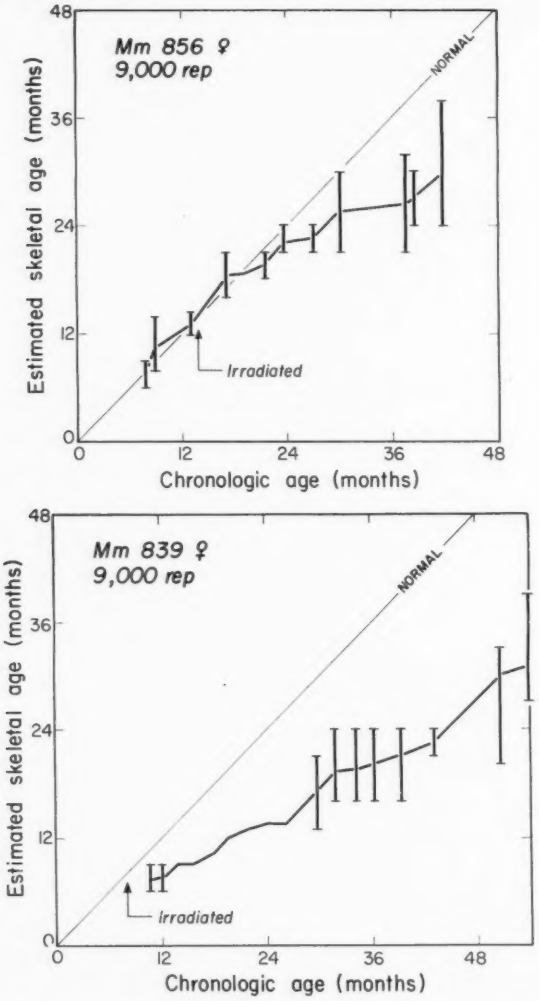


FIG. 12. Increase in sitting height in irradiated females. The menstrual periods and subsequent periods of amenorrhea are shown at the bottom of the figure. Shaded area: control values (one S.D. from mean).

more rapidly growing controls until the time of irradiation when the rate of increase in sitting height was abruptly reduced to approximately half. This rate, which was less than that of slower growing controls, was maintained to the termination of the observation period. In Mm 839 the rate of change in sitting height before irradiation with 9000 rep, and for $5\frac{1}{2}$ months thereafter, was comparable to that of the slower growing controls; subsequently growth was absent for a 10 month period, then resumed at a rate less than half that of the slower growing controls. In Mm 862 the rate of change in setting height before irradiation, and for 15 months following 4500 rep,



FIGS. 13–16. Rate of skeletal ageing of four irradiated female monkeys. The limits of the vertical bars represent the spread of maturation of the particular epiphyses suitable for analysis of differentiation at the given chronological age. The continuous line without bars represents the mean rate of differentiation of the skeleton for females of the colony.

FIG. 13. (upper) Skeletal maturation in Mm 856, 9000 rep.

FIG. 14. (lower) Skeletal maturation in Mm 839, 9000 rep.

paralleled the colony average, then slowed abruptly to half that of the slower growing controls. In the case of Mm 903 routine measurements were not made for 2 years following irradiation with 4500 rep, from which time to the termination of the experiment the rate of increase in sitting height was somewhat less than that of the slower growing controls.

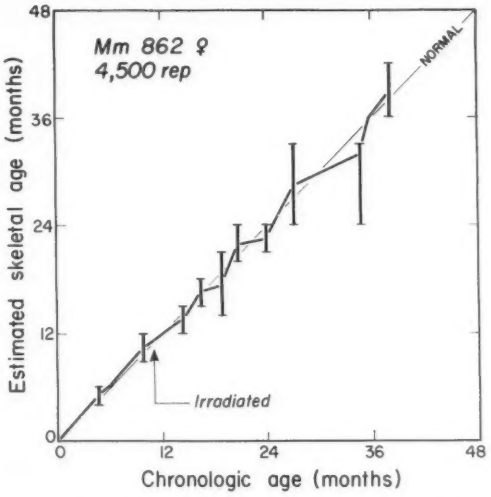


FIG. 15. Skeletal maturation in Mm 862, 4500 rep (see p. 840 for legend).

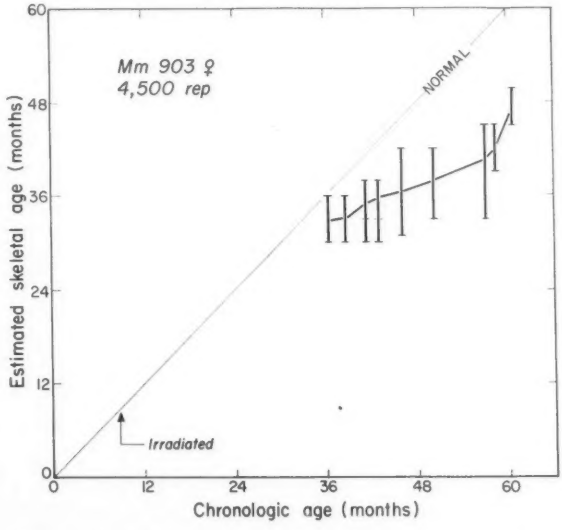


FIG. 16. Skeletal maturation in Mm 903, 4500 rep (see p. 840 for legend).

Skeletal ageing. Using the series of roentgenograms taken at 3 month intervals, graphs were prepared showing the skeletal maturation of each irradiated monkey. We are indebted to C. W. Asling, Department of Anatomy, University of California, Berkeley, for the thorough analyses of status of skeletal development of irradiated monkeys. The skeletal age plotted in relation to the chronological age was then compared with the standard for the colony (5) (Figs. 13–16). In the graphs representing the skeletal ageing, the variations in age estimate introduced by the timing of critical changes

in different bones of the appendicular skeleton are indicated by vertical bars. More variability was encountered in the experimental than in normal animals. In the latter, for instance in the 2 to 3 year age group, there was sufficiently close agreement in age of closure of the various epiphyseal plates so that estimates of bone age could be made within an accuracy of 3 months, whereas in the irradiated monkeys it was difficult to reach decisions on bone age within an accuracy of 6 to 9 months. No irradiated monkey showed total arrest of bony epiphyseal maturation; slight progress was made at virtually every interval of observation in every animal. None of the epiphyses showed clearly the "line of arrested growth" which characterizes full arrest of bony epiphyseal maturation such as occurs in hypophysectomized animals (6, 7, 8); instead there was a diffuse bony shadow representing trabecular bone adjacent to all cartilage plates, indicating the possibility of cartilage erosion.

Of the two females subjected to 9000 rep, skeletal maturation in Mm 856 began to lag within 4 to 9 mo. (Fig. 13); in the second (Mm 839) skeletal maturation was retarded more drastically and sooner, the change being detectable within 3 months after irradiation (Fig. 14). Though maturation of bone was more decisively retarded than in the other irradiated female, some differentiation did occur late in the observation period. In the male, which received 9000 rep, the rate of bony maturation (not illustrated) lagged behind normal soon after treatment and the retardation continued for about 9 months. Late in the observation period, however, in the last 1.5 year, some bony differentiation did occur and by the time of autopsy skeletal age was within normal limits. One of the two females which received 4500 rep (Mm 862) showed normal rate of bony maturation (Fig. 15). The other (Mm 903) was not observed for the first 2 years, but the bony maturity was definitely retarded during the 3rd year, after which some recovery occurred during the 4th year (Fig. 16).

Teeth

The time of eruption of teeth was recorded and the normality of dentition was evaluated by the standards established for this species (9). The time of emergency of permanent teeth was unaffected in the 9 months period after irradiation with either dose administered at ages between 8 mo. and 1 yr. However, deuteron irradiation caused retardation in the emergence of the permanent teeth which were scheduled to erupt after this time. It will be recalled that in the rat, hypophysectomy retarded eruption and growth of incisors (10).

Reproductive System

At the time of autopsy all experimental females were 3 to 5 years of age and therefore beyond the age of menarche. Those which received the higher dose of radiation, 9000 rep, had a primary amenorrhea; they had never menstruated, showed no sex skin changes, and no vaginal desquamation. Those irradiated with 4500 rep reached menarche, one prematurely, the

other late; the menstrual cycles persisted for a short period, 9 and 15 mo., after which there was cessation of periodic bleeding. At the time of autopsy 7.5 and 15 mo. later, these two animals still were amenorrheic (Table 2).

The ovaries of all irradiated monkeys were small, pale and translucent (Fig. 17, 18). Histological examination showed that no follicles greater than 0.5–1 mm. in diameter were present, and there were no corpora lutea or vestiges thereof. There was therefore no evidence in the ovaries that ovulation had ever occurred. The ovaries from animals which had received 9000 rep were comparable in structure to those of 1 to 1.5 year old animals. Many primordial follicles were present, perhaps more than are typical for

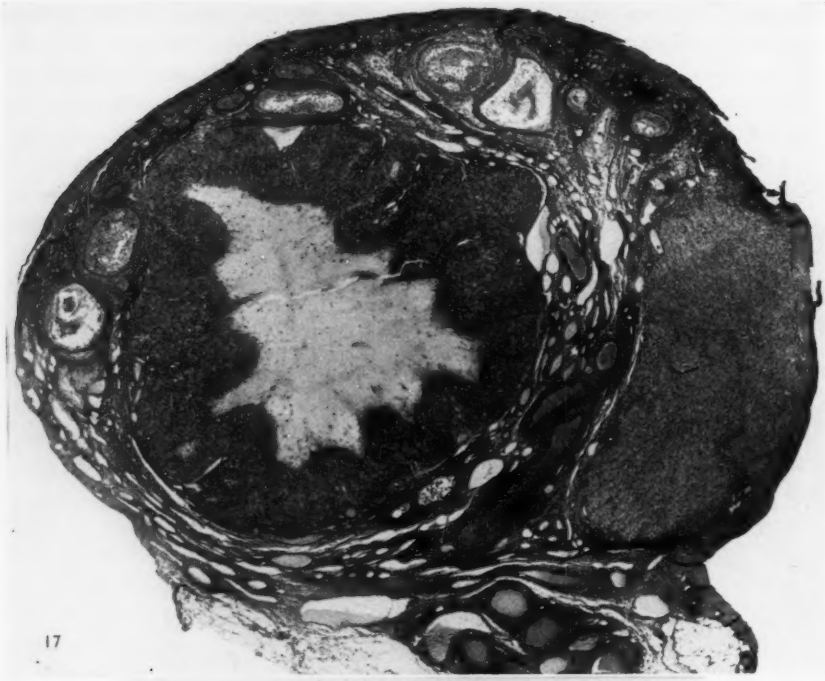
TABLE 2. EFFECT OF DEUTERON IRRADIATION TO THE PITUITARY ON THE REPRODUCTIVE SYSTEM OF THE FEMALE MACAQUE

Dose of Radiation rep	Monkey Number	Age at Menarche* yr.-mo.-da.	Period Menstruation Continued mo.	Period of Amenorrhea mo.	Age at Autopsy yr.	Ovaries Weight mg. L	Ovaries Weight mg. R	Ovaries Structure	Dimensions of Uterus mm.
9000	839	Primary	Amenorrhea		4.5	54	49	small follicles no corpora lutea	8× 3×11
	856	Primary	Amenorrhea		3.5	**	86	small follicles no corpora lutea	10× 6×11
4500	903	3-6-14	9	15	ca 5	86	94	small follicles no corpora lutea	9× 4×13
	862	1-4-27	15	7.5	3.2	**	198	small follicles no corpora lutea	9.5× 3×17
Controls	946	—	Regular	—	ca 3.5	**	288	Growing follicles and corpora lutea	18×14×25
	887	2-2-4	Regular	—	3.5	**	404	"	18×16×20
	695	1-7-1	Regular	—	5.0	**	475	"	18×14×27

* Normal limits for the colony 1-5-5 to 2-7-21.
** One ovary of Mm 862 was removed at 9 mo. of age, of Mm 856 at 1 yr., of controls 1 to 2 years before autopsy. The remaining ovary may be enlarged due to compensatory hypertrophy.

the chronological age. Numerous cords of epithelioid cells were also prominent in the tunica albuginea. In monkeys whose pituitaries had been irradiated with 4500 rep the ovaries contained many polyovular follicles, but after 9000 rep few or no polyovular follicles were present; there were instead more anovular cords in the inner cortex. The ovaries of Mm 839 were the smallest, in keeping with other findings in this animal, indicating that the pituitary had been more seriously damaged than in other monkeys.

The mammary gland development was lacking in monkeys whose pituitaries had received the higher dose, whereas definite duct development was present in those which received the low dose, reflecting the difference in development and functional status of the ovaries. The diameter of the mammary glands after 9000 rep was 0.5 to 1 cm.; after 4500 rep the diameter was 8.5 to 9 cm. comparable to the diameter in the adult multipara (Fig. 8). The latter were, however, not typical of normal adult glands but, rather, of regressed glands (11). The size of these glands indicated that duct development had progressed in a manner to be expected during the establishment of the menstrual cycle, but there was a deficiency in the alveolar component. The glands consisted of narrow branching ducts with



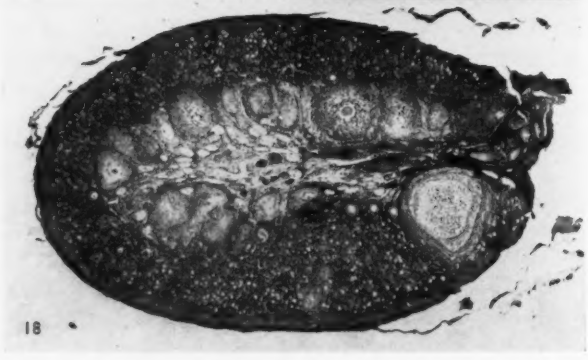


FIG. 17. Ovary Mm 857, control, adult ovary containing corpora lutea of two ages. Age 3 yr., 6 mo., 22 da.

FIG. 18. Ovary Mm 839, 9000 rep, wide cortex, small follicles, no evidence of old corpora lutea, age 4-6-5. H & E ×17.

little or no associated acinar tissue, the pattern being similar to that found in the sexually mature monkey deprived of ovarian function by castration. The mammary glands of the monkeys treated with 9000 rep had not developed beyond the basic size characteristic of the very young male or female, or the adult male. They could be distinguished from young growing

glands by the absence of terminal buds on the short tapered ducts. The nipple size paralleled closely the mammary gland size. As can be noted in photographs showing the ventral surfaces of the monkeys the nipples had developed in the larger animals which received the lower dosage, but not in those whose growth had been most seriously impaired by the higher dose (Fig. 7).

The uteri were small in all four irradiated monkeys, being comparable in dimensions to those of prepuberal monkeys (Table 2).

The male whose pituitary was irradiated with 9000 rep, and its control were still sexually immature at the time of autopsy when 2 yr. 2 mo. of age. Even at this early age the testes of the irradiated male were smaller and less mature than in the control (testis 290 mg. compared with 812 mg. in control).

Changes in Endocrine Organs and Other Viscera

The *adrenals* were much smaller in irradiated animals than in controls, perhaps out of proportion to the reduction in body weight. The organizational pattern of the adrenal cortex was less distinct in irradiated monkeys than in controls. Beside reduction in size of the cortical cells, the most consistent change was the reduction in width of the zona reticularis. This was more pronounced after the higher dose of radiation. A distinct subglomerular lipid free zone, such as characterizes the adrenal cortex of the hypophysectomized rat, was not seen in adrenals of irradiated female monkeys, and was only suggested in that of the irradiated male. The amount of lipid and the droplet size were not markedly changed in the zona fasciculata; the lipid content of zona glomerulosa and zona reticularis was low in adrenals of control as well as of irradiated monkeys. Though the weights of the adrenals were within the range described by Knobil *et al.* (12) in the surgically hypophysectomized monkey, the histological changes were not so pronounced.

The *thyroids* were much smaller in irradiated than control monkeys, and were within the weight range found after hypophysectomy, but they were not histologically distinctive. In all thyroids, including those of the controls, the epithelium was low and the colloid compact. A limited number of observations were made of the I^{131} uptake, and as shown in Table 3 the percent uptake of the injected dose was low compared with pre-irradiation values. However, the uptake remained much higher than would be anticipated after complete hypophysectomy.[4]

[4] No data were found in the literature, on iodine uptake by thyroids of hypophysectomized monkeys. However, E. Knobil by personal communication stated that no iodine uptake could be detected in the hypophysectomized monkeys examined. He gives the following value for surgically hypophysectomized monkeys of about 4 kg. B.W., 1–7.5 mo. after operation: thyroids 205 to 472 mg., average 315 mg. compared with 436 mg. in controls; adrenals 245 to 410 mg. average 321 mg. compared with 633 mg. in controls.

The *parathyroids, thymus* and *spleen* showed no consistent alterations. The *viscera* from irradiated monkeys: spleen, lung, heart and liver weighed less than those of normal controls, roughly paralleling the decreased body size. No histological changes attributable to irradiation were present in these organs, though the decreased organ weights were at least in part due to decreased cell size (Table 3).

White and Red Blood Cells

Total and differential counts of leukocytes, total red cell counts, and determinations of hemoglobin and hematocrit were made at intervals during the period of observation. Total circulating red cell volumes were determined at the beginning and end of the experiment. On the basis of these somewhat limited data no definite change was demonstrated in blood values.

Brain Injury

Careful analysis of the brains was made by J. Cammermeyer and W. F. Windle of the Laboratory of Neuroanatomical Sciences, National Institutes of Neurological Diseases and Blindness. The brains of the four irradiated female monkeys were perfused *in situ* with acacia-formalin; embedded in paraffin. Serial sections were made of the infundibulum and representative sections from other areas. They were stained with gallocyanin chrome alum. Gross examination showed that the leptomeninges in all brains were moderately thickened around the optic chiasm and particularly in the region of the infundibulum. Greater thickening was present in the animals which had received the higher dosage of irradiation. In these animals, also, the intima of the small vessels of the leptomeninges around the infundibulum were thickened and hyalinized.

Despite the extensive destruction which had occurred in all irradiated pituitaries the damage to the posterior lobe and infundibulum was sufficiently severe to cause retrograde degeneration of the associated hypothalamic nuclei in only one of four brains. The paraventricular and supraoptic nuclei were intact in both animals which had received 4500 rep and in one which had received 9000 rep; in the other (Mm 839) there was a marked atrophy and loss of cells in the paraventricular, and particularly in the supraoptic nucleus. This was the only brain in which the adjacent parts of the cerebrum were damaged. This damage in the hypocampal gyrus was greater on the right side, though there was also minimal damage, with a pinpoint area of necrosis, on the left side. The areas of coagulation necrosis were delimited by glial membranes, indicating that the lesions were of long standing. In the cortex adjacent to the area of coagulation-necrosis on the right side, and over the small pinpoint necrosis on the left, there was a diffuse area where the astrocytes were hypertrophied and where there was, in addition, focal loss of the pyramidal cell layer. The

TABLE 3. ORGAN WEIGHTS OF DEUTERON-IRRADIATED MONKEYS AND THEIR CONTROLS

Sex	Animal Number	Dose rep	Age yr.	BW kg.	S.H. cm.	Adrenals mg.	Thyroid			Pituitary mg.	Thymus gm.	Spleen gm.	Brain gm.	Lungs gm.	Kidneys gm.	Heart gm.	Pancreas gm.	Liver gm.	GI Tract gm.
							mg.	I^{131}% Uptake											
								Onset	Final										
♀	Mm 856	9000	3.5	4.47	45.2	390	186	34.5	16.2	26.1	3.7	3.1	108	23.4	13.7	19.9	—	91	
	Mm 839	9000	4.5	3.20	41.5	443	244		31.5	12.4	2.6	1.6	87	14.1	9.5	10.5	2.6	55	
	Mm 862	4500	3.2	5.09	43.7	484	315	23.7	15.3	25.1	4.1	6.6	92	27.7	16.3	19.4	8.0	110	
	Mm 903	4500	5.0	6.09	47.4	547	208		16.7	23.0	2.2	3.1	87	27.5	13.3	15.8	7.6	98	
	Mm 887	Control	3.5	5.34	46.6	858	633			46.6	1.8	7.6	86	29.2	16.2	21.7	5.2	122	
	Mm 946	Control	ca 3.5	5.20	46.2	848	726			62.1	3.3	5.5	73	35.9	15.3	15.7	4.8	92	
	Mm 695	Control	5.0	6.4	49.6	657	782			81.5	2.4	4.5	92	33.0	16.0	26.0	8.8	121	
♂	Mm 837	9000	2.2	2.62	39.0	590	352			14.3	2.0	2.7	115	52.0	21.3	15.0	4.1	105	103
	Mm 838	Control	2.2	3.97	42.2	876	686			54.2	9.4	8.4	107	75.0	42.2	26.0	9.4	125	190

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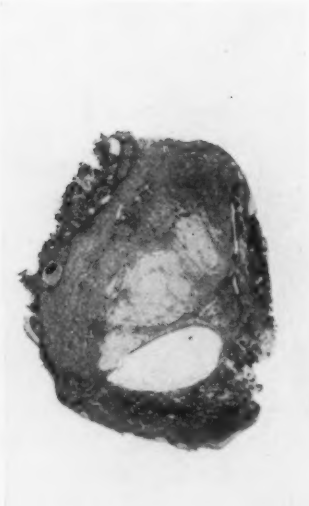

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FIG. 19. Para-sagittal section through pituitary of control monkey (Mm 946, female, age 3.5 yr., B.W. 5.20 kg.). (Posterior lobe left; anterior lobe right.) Periodic acid Schiff (PAS) ×17.5.

FIG. 20. Sagittal section through pituitary of control monkey (Mm 838, male, age 2-2-3, B.W. 3.97 kg.). (Posterior lobe, left.) The distal part of the stalk is deeply embedded in the parenchyma of the anterior lobe. PAS ×17.5. ⟿

leptomeninges overlying the damage showed reactive changes of the vessels. With these few exceptions, restricted largely to one irradiated animal, the damage to the brain was negligible.

Changes in Irradiated Pituitaries

All irradiated and control pituitaries were studied histologically. They were fixed initially in formol (since perfusion was used to harden the brain) and only secondarily in Regaud's fluid. The pituitaries were dissected from the sella turcica after fixation. Some difficulty was encountered in this dissection due to the thickening of the enclosing membranes, and in some instances due to the necrosis of the bony sella turcica. The pituitaries were bisected after fixation and one half of the gland was then sectioned in the sagittal plane and the other half at right angles, in either the horizontal or the frontal plane. After nitrocellulose embedding, the pituitaries were cut at four micra and representative sections from five levels were stained with Mallory-azan and periodic acid Schiff stains.

In the anterior lobe of the glands of normal monkeys, the density of distribution of the two types of chromophils is not constant. In the part of the anterior lobe immediately adjacent to the intermediate lobe the predominant chromophil is the acidophil which stains with orange G after Mallory-azan. The basophils are far more numerous in the anterior portion of the gland. In the antero-superior region the cells of the anterior lobe blend with those of the intermediate lobe to form a sleeve which surrounds the infundibulum. Both acidophils and basophils are distinguishable in this extension of glandular tissue upward toward the brain. Elsewhere in the pituitary a sharp line of demarcation exists between the anterior and intermediate lobes. In addition to the two classical types of chromophils, the basophils staining with aniline blue and acidophils which stain with orange G, there is a third type of chromophil, seen in Mallory-azan stains of the monkey pituitary, which stains deeply with azo-carmine. These carminophilic cells are usually larger than other acidophils, show discrete granules and have a prominent Golgi apparatus which is situated at a distance from the nucleus. The carminophils are readily seen in Mallory-azan stained sections in the predominantly basophilic areas, where they appear both singly or in small groups. They are present elsewhere in the anterior lobe but in lesser numbers. In the periodic acid Schiff stain, these cells, like the eosinophils, take the orange G stain, i.e., do not stain with basic fuchsin, and therefore are PAS negative.

FIG. 21. Sagittal section of irradiated monkey pituitary (Mm 839, female, age 3-9-27, B.W. 3.20 kg., 9000 rep at age 0-8-9). The normal structural pattern is disturbed, with considerable destruction of central portion. Posterior lobe, left; anterior lobe tissue, right Large mass of bone marrow is in direct contact with parenchyma (upper left). Mallory-azan (M-A) ×17.5.

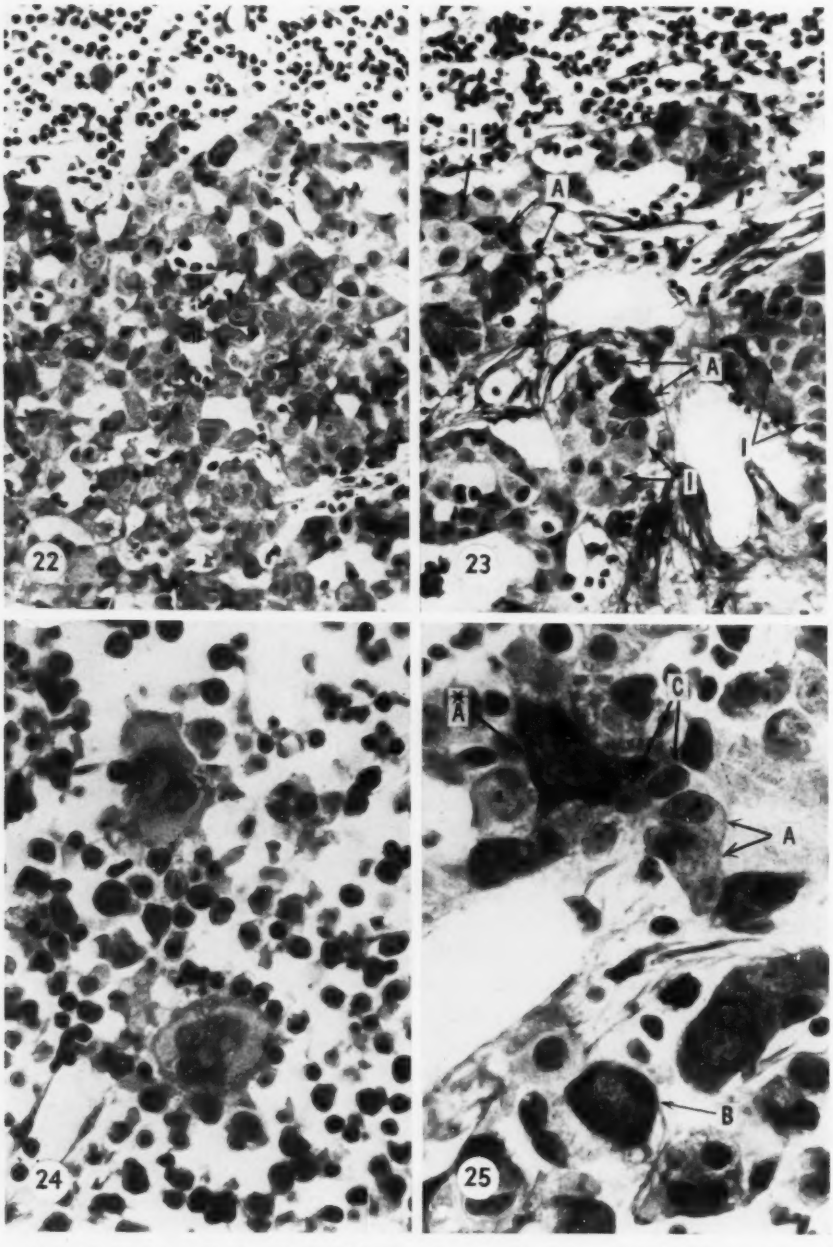


FIG. 22. Anterior pituitary (Mm 839). Due to resorption of part of the sella turcica and adjacent covering membranes, invasion of anterior pituitary by bone marrow has oc-

The intermediate lobe is composed of uniformly sized cells, which do not stain intensely and have vesicular nuclei with small, though prominent, nucleoli. Laterally and posteriorly the intermediate lobe spreads as a thin layer of cells over the neural lobe. The neural lobe of the monkey is relatively large, occupying a large portion of the sagittal section (Fig. 19, 20). The distal part of the infundibulum is embedded deeply in the anterior lobe tissue, and forms an abrupt angle with the part extending upward toward the brain.

There were multiple changes in the irradiated pituitaries, some of which were common to all. The pituitaries from experimental animals were considerably smaller than normal (Table 3, Fig. 21). The reduction in size involved all lobes. The covering membranes were thickened and firmly attached to the gland itself by collagenous fibers which extended into the parenchyma (Fig. 28). Due to such adhesions, the thin peripheral layer of the pars intermedia surrounding the pars posterior was frequently lost in removal of the irradiated pituitaries from the sella turcica.

The general pattern of microscopic structure of the irradiated pituitary was usually still distinguishable even with low magnification; also, the usual distribution of specific cell types was maintained in most of the anterior lobes. Cell size was, however, not so uniform as in control pituitaries. All lobes, especially the intermediate lobe, contained areas where extensive cell destruction or vacuolation of parenchymal cells was widespread (Fig. 26). The walls of the blood vessels were frequently thickened, especially in those near the periphery of the glands (Fig. 29). The reduced cell size, and the partial degranulation which so frequently characterized irradiated glands, may have been secondary to reduced vascularity resulting from such sclerosis. Scar tissue appeared to have replaced parenchyma in areas where small localized hemorrhages had occurred secondarily to damage of blood vessels (Fig. 30). The types of change observed were present in all lobes, and the cellular abnormalities did not affect preferentially any particular cell type or location. The cellular changes involved both cytoplasm and nucleus. Vacuolation was present in cells of different types and in dif-

curred (upper part of field). Blood sinuses are continuous between bone marrow and pituitary tissue. M-A ×320.

FIG. 23. Another section of the pituitary of Mm 839. Pars intermedia (I) and pars anterior (A) have been subdivided into islands by heavy scar tissue. Bone marrow is invading both types of tissue. M-A ×320.

FIG. 24. Bone marrow in area of invasion shown in the preceding figures (Mm 839). Developing leukocytes and erythrocytes are present, as well as megakarocytes. PAS ×500.

FIG. 25. Section through anterior lobe (Mm 839) showing that regardless of extreme damage to the parenchyma all specific cell types are still recognizable. Basophil (B), acidophils stained with orange G (A), or azo-carmine carminophils (A*), chromophobe (C). M-A ×800.

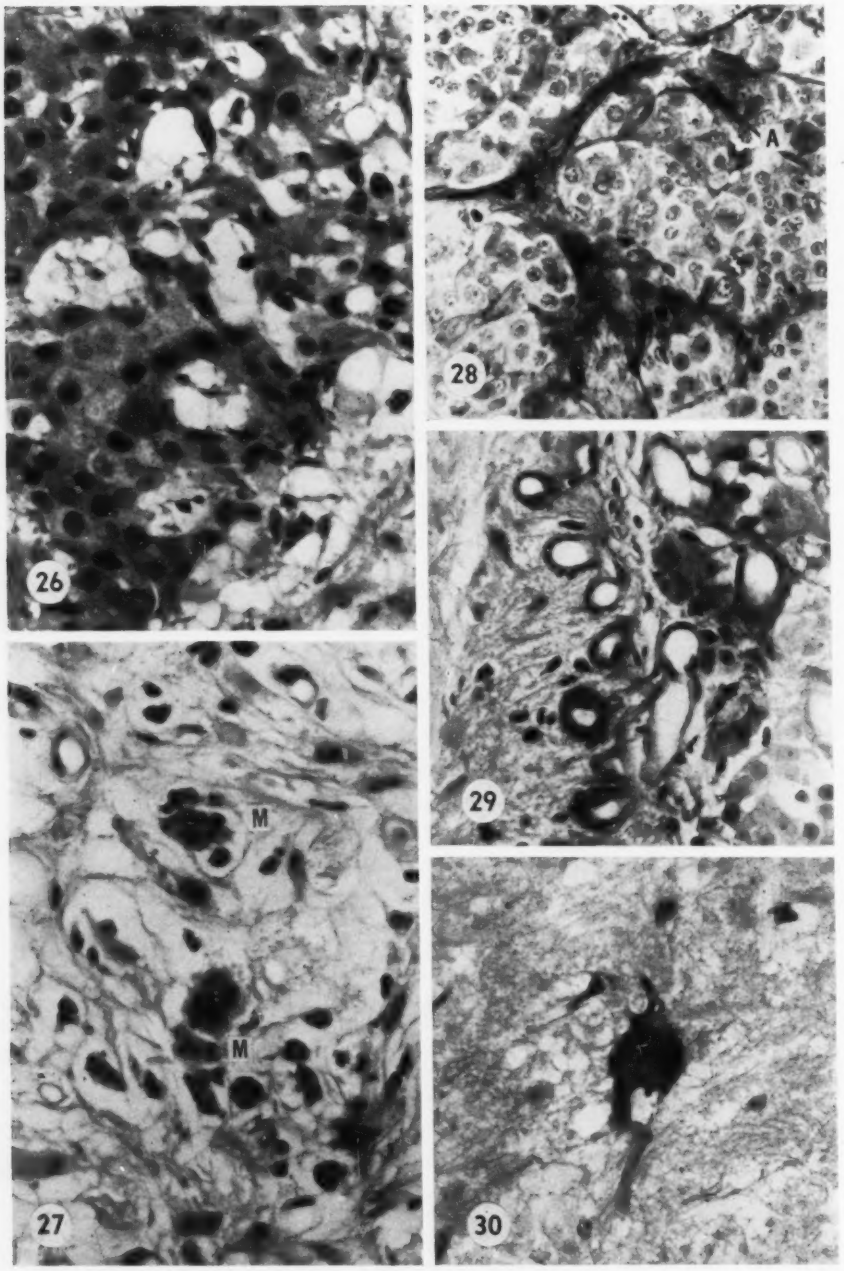


FIG. 26. Intermediate lobe showing damage due to irradiation (Mm 862, female, 2-3-27 B.W. 5.09 kg., 4500 rep at 0-9-6). The cytoplasm of surviving cells is deeply stained and

ferent parts of the gland (Fig. 34 to 39). Cells of unusually large size were found in all lobes of the hypophysis and among all types of parenchymal elements (Figs. 33 to 39). Abnormal mitotic divisions were found in all parts of the pituitary (Figs. 31, 32). Abnormal mitoses with resultant abnormal cells have also been described in irradiated hypophyses of rats (1, 2). No adenomata were visible, either grossly or microscopically in irradiated monkey pituitaries and there were no nests of abnormal cells, as found in rat pituitaries at low radiation doses and after long post-irradiation periods (2).

The changes in the pituitary of monkey 839 (9000 rep) require a more detailed description. This pituitary was the smallest of the experimental glands, weighing only 12.4 mg., and was so altered that it was impossible to distinguish the separate lobes with low magnifications (Fig. 21). The central portion of the gland was composed of homogeneous material interspersed with dense strands of connective tissue. Small cysts and localized hemorrhages were present among the surviving parenchymal cells; only with higher microscopic resolution could the tissue be identified as part of a particular lobe. There was evidence that multiple small hemorrhages had occurred and were still occurring at time of autopsy; well preserved red blood cells were found free in the tissues in certain areas, as well as macrophages enclosing hemosiderin (Fig. 27). The remaining parenchymal cells were grouped in islands or cords by scar tissue (Fig. 23). It was noted at the time of removal of the pituitary that part of the sella turcica had been destroyed and in certain places the bone marrow had actually invaded the anterior and intermediate lobes (Figs. 21 to 23). Thin walled sinuses could be seen passing from one tissue to another without interruption. Various precursors of leukocytes and erythrocytes, as well as megakaryocytes, could be identified within the parenchyma, making it conclusive that this invading tissue was bone marrow (Fig. 24). Regardless of the profound alterations in all parts of the pituitary and the many abnormal cell forms present, all types of chromophils were still recognizable. As far as could be judged from morphology, these cells were functional; their Golgi apparatus

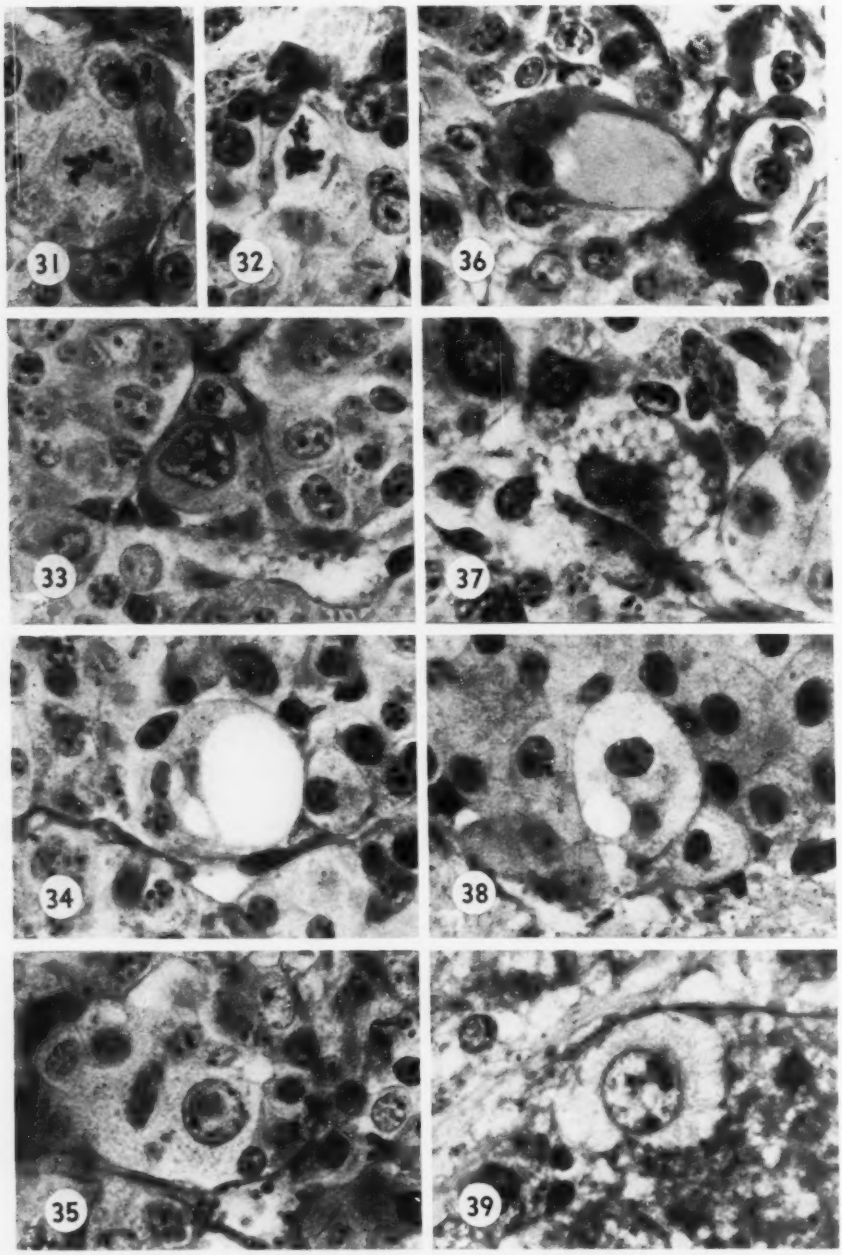
nuclei are pyknotic. Some cells are extrensively vacuolated. Cell size is variable. M-A ×500.

FIG. 27. Changes in the posterior lobe following irradiation (Mm 839). Scar tissue has distorted the organization. Macrophages (M) still contain ingested matter, chiefly crystals of hemosiderin. M-A ×500.

FIG. 28. Anterior lobe (Mm 856, female, age 3-5-19, B.W. 4.47 kg., 9000 rep at 1-1-24) showing excessive proliferation of collagenous fibers. Parenchymal cells are small, and most are degranulated. A well granulated acidophil is marked (A). M-A ×320.

FIG. 29. Thickening of the walls of blood vessels in periphery of the pituitary (Mm 862). M-A ×450.

FIG. 30. Avascular scar tissue replacing part of posterior lobe tissue (center) (Mm 862). M-A ×500.



FIGS. 31–39. Various atypical pituitary parenchymal cells. Note the similarity between abnormalities present after irradiation in cell types of different lobes. All photomicrographs are from the pituitary of Mm 856, except fig. 38 which is from Mm 862.

was often enlarged and normal granulations and normal staining reactions characterized them (Fig. 25).

DISCUSSION

There was no evidence from morphology for differential radio-sensitivity of any of the anterior pituitary chromophils at this post-irradiation period. Haymaker *et al.* (13) described destructive changes in the basophils in monkey pituitaries within a few hours after irradiation with 5000 to 10,000 r from gamma particles. In the rat a slightly greater resistance of the gonadotrophic basophils was reported after low dosage and long post-irradiation periods (2).

All functions of the pituitary were depressed. The reproductive system was hypofunctional in every instance. In the two females subjected to 9000 rep there was a primary amenorrhea and the ovaries, uterus and vagina remained infantile long after the time of normal menarche; the mammary gland and nipple up to 4.5 years of age remained in the condition characteristic of the immature male or female. The two monkeys whose pituitaries were irradiated with 4500 rep reached menarche, and irregular menstrual cycles continued for 9 and 15 mo., after which secondary amenorrhea persisted until autopsy 7.5 to 15 months later. Growth, as measured by body weight, sitting height, and skeletal differentiation was impaired in all the monkeys whose pituitaries were irradiated, but growth stasis occurred only in two of the three animals irradiated with 9000 rep (Mm 839, Mm 837). In general, skeletal differentiation paralleled body growth. The adrenals were reduced in weight, and the cortex was narrower, chiefly due to atrophy of the zona reticularis. The atrophy of this zone was more complete in the animals whose pituitaries were irradiated with 9000 rep. The thyroids of irradiated animals were one half to a third normal weight, but histological differences were not detected. Study of the I^{131} uptake showed that they were still capable of incorporating a large part of the injected dose.

FIGS. 31–32. Anterior lobe—abnormal mitoses in chromophobes (no stainable granules were present). M-A ×800.

FIG. 33. Anterior lobe—basophil with atypical nucleus (PAS positive granules present). ×800.

FIG. 34. Anterior lobe—chromophobe, enlarged and vacuolated. No stainable granules were present. There are two smaller cells embedded in its cytoplasm. M-A ×800.

FIG. 35. Anterior lobe, acidophil partially depleted of granules. Nucleus contains a large vacuole. The outlines of the cell are not distinct due to engulfed chromophobes. PAS ×800.

FIG. 36. Anterior lobe—enlarged cells containing basophilic granules and single vacuole (signet ring type). M-A ×800.

FIG. 37. Anterior lobe—enlarged basophil with multiple vacuoles and a few residual basophilic granules. M-A ×800.

FIG. 38. Intermediate lobe—enlarged and vacuolated cells. PAS.

FIG. 39. Posterior lobe—enlarged vacuolated cells. M-A ×800.

It appears, therefore, that of the pituitary target organs and functions, the gonads were most sensitive to pituitary damage. In females which were irradiated with 4500 rep, reproductive function was suppressed after a delay, whereas after 9000 rep regression occurred sooner. Body growth was somewhat less sensitive to pituitary damage; though slowing of growth occurred after 4500 rep and there was also retardation of skeletal differentiation, complete suppression did not occur even after 9000 rep. The effect of pituitary irradiation on the adrenals was slight after 9000 rep; the least effect was observed on the thyroids.

In the rat, "evidence for diminished hormone secretion from the anterior pituitary appeared in the dependent endocrine function in the following sequence, as the dose or time interval increased: adrenal atrophy with decreased cortical width, thyroid atrophy, slowed body growth and finally atrophy of the testes and dependent accessory sex organs," (2). It was only after irradiation of the rat pituitary with 25,000 rep or more that the organization of the pituitary was obliterated, only a few epithelial cells remaining at the periphery of the gland, and that immediate cessation of endocrine functions occurred.

No tumors were noted in the irradiated monkey pituitaries though the glands were examined as late as $4\frac{1}{3}$ years after irradiation. Adenomatous changes had been found to be frequent in the irradiated pituitaries of rats (2), after a much briefer period (2 yr.). It should be noted, however, that adenomas were only observed in pituitaries of rats irradiated with the low doses (945 and 1575 rep), none being observed at doses of 3150 to 6300 rep, doses more nearly comparable to those applied to monkey pituitaries.

A considerable time intervened between irradiation of the monkey pituitary and the expressed effects of radiation. At the higher dose the depression of growth rate was immediate or became evident within 6 mo. whereas after the lower dose retardation in growth did not appear for over a year. The time-lag was also shown clearly by the reproductive system of the two females subjected to the lower dosage of pituitary irradiation; after establishment of menstrual cycles, secondary amenorrhea supervened 2 to $2\frac{1}{2}$ years after irradiation; implying delay in failure of pituitary support. Such a delay in appearance of the effects of pituitary irradiation had been shown clearly in the study of the rat. After irradiation with 6300 rep atrophic changes were observed within 3 months, in the pituitary and all its target organs, and body growth was depressed. After 12,600 rep all were affected within 1 to 2 months. Immediate cessation of pituitary function did not follow until doses of 20,000 rep or more were given.

No evidence of functional recovery was observed in the rat. There was evidence in the present study that limited recovery of the pituitary occurred several years after irradiation. The monkey in which the pituitary had been most severely damaged following irradiation by 9000 rep, failed to increase in body weight and length for a 10 month period and then re-

sumed growth at a slow rate. This observation suggests that at least a small degree of recovery had taken place in the pituitary.

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NOTES AND COMMENTS

EFFECT OF PITUITARY GROWTH HORMONE ON LYMPHATIC TISSUES, THORACIC DUCT LYMPH FLOW, LYMPH PROTEIN AND LYMPHOCYTE OUTPUT IN THE RAT[1]

ABSTRACT

Administration of beef anterior pituitary growth hormone to hypophysectomized rats produced increases (out of proportion to body weight gain) in weights of thymus, spleen and mesenteric lymph nodes and in total lymph protein and lymphocyte output from the thoracic duct. Cervical lymph nodes were enlarged in direct proportion to body weight changes.

Certain lymphatic tissues are increased in weight by growth hormone treatment (reviewed by Dougherty (1), Lundin (2)). The present experiments were planned to correlate changes in lymphatic tissues (thymus, lymph nodes, spleen) with possible alterations in protein and cell output of thoracic duct lymph, and in the peripheral white blood cell levels.

METHODS

Fourteen male rats, hypophysectomized at the age of 60 days, were maintained for 30 days on Diet I,[2] wet, with lettuce, after which time daily injections of beef anterior pituitary growth hormone[3] were begun in seven rats and continued for 40 days. The hormone was administered by intraperitoneal injection of 100 μg. in saline solution. Control animals were injected with an equal volume of 0.9% saline solution. The potency of the hormone was such that daily injections of 10 μg. for 10 days, in rats hypophysectomized at the age of 28 days, gave a 13 gm. body weight increment. A total dose of 10 mg. of this preparation (3) over a four day period gave no histological evidences of ACT, TSH or FSH in hypophysectomized female rats. Prior to autopsy, duplicate white blood cell counts were obtained from tail vein blood. The rats were anaesthetized with sodium pentobarbital (7 mg./100 gm. B.W.) and thoracic duct lymph was collected (4) for a one hour period. Cell counts and micro-Kjeldahl estimates of total lymph protein were obtained. Lymph nodes, thymus and spleen were excised, cleaned and weighed. Data were analyzed using Fisher's method for small samples. P values of $<.01$ are considered to indicate significant differences.

RESULTS AND DISCUSSION

On either a relative or absolute basis, thymus and mesenteric lymph nodes were enlarged following growth hormone treatment (Fig. 1). The spleen underwent a less significant enlargement. Cervical lymph nodes increased in weight, but only in proportion to the increment in body weight.

Figure 2 shows body weight increase following growth hormone treatment, and presents the data showing increases in lymph flow, lymphocyte output and lymph protein content, as calculated for a 24 hour period.

There were no significant changes in either total or differential blood leucocyte counts in these animals as the result of treatment with growth hormone.

These data are interpreted as indicating that beef pituitary growth hormone is effective in producing an increase in weight of thymus, spleen, mesenteric lymph nodes, and in increasing the volume of lymph flow, total lymphocyte output, and thoracic duct

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[1] Supported in part by grants from the USPHS and the Santa Clara County Heart Association.

[2] For composition of Diet I, see Endocrinology 48: 537. 1951.

[3] Beef pituitary growth hormone was furnished by C. H. Li.

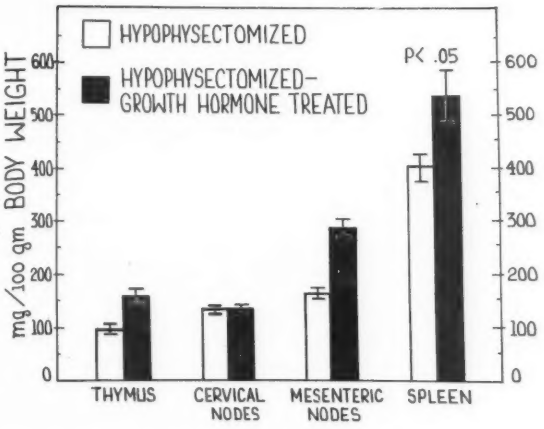

FIG. 1. Relative weight changes of thymus, spleen, cervical and mesenteric lymph nodes, in hypophysectomized rats treated with growth hormone. The vertical bars show ±1 S.E.M.

lymph protein output in hypophysectomized animals treated over a relatively long term. Of the tissues examined, the cervical lymph nodes responded only in proportion to body weight increase. This difference in response has been noted in previous experiments in which growth hormone was administered to hypophysectomized rats paired-fed with controls for 10 day periods (5). In these experiments, the thymus and spleen enlarged out of proportion to the body weight increase, whereas the cervical lymph nodes did not.

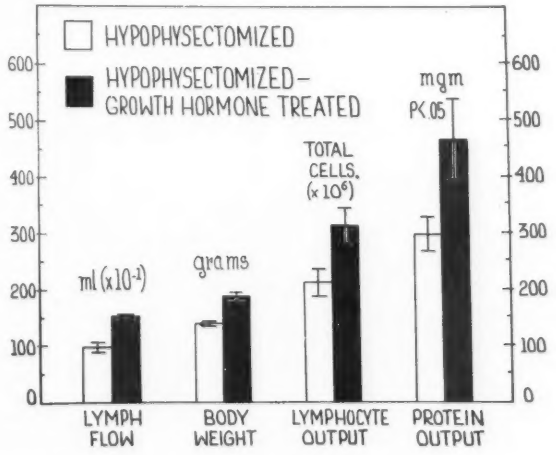

FIG. 2. Increases in thoracic duct lymph flow, lymphocyte output and lymph protein output (calculated for a 24 hour period), in hypophysectomized rats treated with growth hormone. The vertical bars show ±1 S.E.M.

The present experiments show that the increases in size of the visceral lymphatic tissues are accompanied by increases in thoracic duct lymph flow, lymphocyte output and lymph protein output. It is suggested that these changes reflect an increased fluid and protein exchange and an increased output and possibly increased utilization of lymphocytes in the body as a consequence of growth hormone treatment. In this sense, these representative visceral lymphatic tissues may be target organs for growth hormone, with the implication from prior experiments (5) that the changes noted are not necessarily dependent on increased food intake.

MARVIN M. SHREWSBURY

Department of Biology
San Jose State College
San Jose, California

WILLIAM O. REINHARDT

Department of Anatomy
School of Medicine
University of California
San Francisco

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ABSENCE OF AN EXOPHTHALMOGENIC SUBSTANCE IN TRANSPLANTABLE THYROTROPHIN PRODUCING TUMORS OF THE PITUITARY OF MICE

ABSTRACT

No exophthalmogenic activity could be demonstrated in extracts containing large amounts of TSH from transplantable pituitary tumors of mice. This indicates that TSH is different from EF.

It has been known for a long time that extracts of the pituitary of stockyard animals produce exophthalmos in a variety of species. However, it is not known with certainty that the exophthalmogenic factor (EF) in pituitary extracts is different and separable from the six well-recognized anterior pituitary hormones, especially thyrotrophin (TSH). Dobyns and Steelman (1) have presented evidence that partial separation of the exophthalmogenic and thyrotrophic properties of pituitary extracts can be achieved with 8% trichloracetic acid. The purpose of this note is to present more conclusive evidence for the non-identity of EF and TSH. The evidence was obtained by an examination of the EF and TSH activities of extracts of transplantable pituitary tumors in mice.

Lyophilized transplantable TSH producing pituitary tumors of tumor strain 77D (2) were extracted by percolation (3). The TSH activity of the extract was measured by thyroidal I^{131} depletion in baby chicks (4) and expressed as U.S.P. units. EF activity of the extract was measured in Fundulus heteroclitus by observing the extent and severity

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TABLE 1. THE ABSENCE OF EXOPHTHALMOGENIC FACTOR IN TRANSPLANTABLE PITUITARY TUMORS IN MICE

	TSH			EF
	Potency	Dosage		Response
	u/mg.	μg.	mu.	units
Mouse tumor extract	1.5	3,000	4,500	0
Thyrotropar	1.0	10	10	1

of the exophthalmos produced in groups of five fish following a single injection and expressed as arbitrary exophthalmogenic factor units (EFU) (5).

The extract obtained by percolation of the mouse pituitary tumors was found to contain 1500 U.S.P. mu. TSH per mg. This extract did not produce exophthalmos in Fundulus in doses as large as 3 mg., or the equivalent of 4500 U.S.P. mu. of TSH per fish (Table 1). That this negative result was not due to the insensitivity of the fish was demonstrated by control experiments in which a commercial preparation of bovine TSH (thyrotropar, Armour and Company) was injected. The equivalent of 10 U.S.P. mu. TSH of this preparation (or 10 μg.) was found, at the time of these experiments, to represent approximately 1 EFU. In terms of TSH, therefore, a dose of the tumor extract which was 450 times greater than that of crude bovine TSH (thyrotropar), did not produce exophthalmos.

The inability of a potent thyrotrophin preparation from mouse pituitary tumors to produce exophthalmos cannot be ascribed to some unique species specificity. Serial section of the floor of the pharynx of Fundulus gave histologic evidence of stimulation of the thyroid in animals injected with mouse tumor TSH extract. Thus, the thyrotrophin of mouse pituitary tumors was effective on the thyroid of the fish, and, it is unlikely that any EF in the same extract would be ineffective on the orbital tissues of the same animal because of species specificity. It appears that the inability of the mouse pituitary tumor extract to produce exophthalmos is due to the absence or virtual absence of EF in the thyrotrophin-rich pituitary tumor.

R. W. BATES, A. ALBERT[1] AND P. G. CONDLIFFE

National Institutes of Health
and
Mayo Clinic and Mayo Foundation
Rochester, Minnesota.

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APPLICATIONS FOR TRAVEL ALLOTMENTS 1st INTERNATIONAL CONGRESS OF ENDOCRINOLOGY

The 1st International Congress of Endocrinology will be held in Copenhagen, Denmark, July 18th to 23rd, 1960. The Endocrine Society has obtained funds to provide a limited number of allotments in support of travel to the Congress for scientists residing in the United States and Canada who may require such assistance. Individual allotments will not exceed $750.00 each.

Application for travel allotment should be submitted in duplicate in the form of a letter giving information on age, training, publications, academic or professional title and society affiliation. Applicants who plan to submit papers for presentation at the Congress should include the proposed title of each paper. Such letters should be submitted *before November 15, 1959*, and should be addressed to Dr. H. H. Turner, Secretary, The Endocrine Society, 1200 North Walker, Oklahoma City 3, Oklahoma. Applicants will be responsible for their own passports, registration, travel arrangements and hotel reservations.

Application for assistance towards travel expenses are entirely separate from applications for registration for the Congress and for submission of papers, information on which may be obtained by writing to The Secretariat, Hormonafdelingen, Statens Seruminstitut, Copenhagen.

CONGRESS OF THE PAN AMERICAN MEDICAL ASSOCIATION MEXICO CITY, MAY 2–11, 1960

Section on Endocrinology

Authors wishing to present papers are invited to submit titles and an abstract in triplicate, of not more than 250 words. Abstracts must be informative and must include results and conclusions. Please indicate, on one copy of the abstracts, whether you will need a slide projector, and what size ($3\frac{1}{4} \times 4$ or 2×2). Papers will be limited to 15 minutes. Technical information regarding hotel reservations, transportation, etc., will be announced by the Pan American Medical Association shortly. Please send abstracts to the program chairman Dr. Karl E. Paschkis, Jefferson Medical College, Philadelphia 7, not later than December 31, 1959.